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PRINCIPAL INVESTIGATOR: Linda Z. Penn, Ph.D.

CONTRACTING ORGANIZATION: University Health Network
Toronto, Ontario M5G 2C4 Canada

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Introduction

Background: Deregulation of the cellular myc proto-oncogene is one of the strongest activators of tumorigenesis and understanding the target genes and pathways regulated by this transcription factor in cancer etiology will clearly mark a key advance.(1,2) Myc expression and activity are highly restricted in normal human mammary epithelial cells (HMECs), but unleashed and deregulated in cells of malignant transformations. Because the protein product is identical in normal and tumor cells, three major issues arise. First, a definitive approach to detect oncogenic Myc in primary tumor specimens is severely lacking and long overdue. This issue has plagued the field during the two decades since Myc was first discovered. Second, it remains unclear whether Myc *function* is different in normal and tumor cells. Myc may regulate the same subset of target genes in both settings, but in a more robust manner in tumor cells. By contrast, deregulated, overexpressed Myc protein may bind and regulate an additional unique set of target genes in tumor cells. This issue has not yet been explored. Third, although it is clear that inhibiting Myc can trigger tumor regression and eradication in animal models, few initiatives are underway to target Myc as a therapeutic approach for human disease. Because Myc protein in normal and tumor cells is indistinguishable, it is thought that anti-Myc inhibitors would have little to no tumor specificity or therapeutic index. Clearly a novel approach is required.

Hypothesis: Our 'idea' is that in addition to the target genes regulated by Myc in non-transformed cells, constitutively activated and overexpressed Myc protein in tumor cells will directly bind and regulate a unique set of target genes that directly contribute to the carcinogenic process. For example, at high levels of expression, Myc may bind low affinity sites and regulate a distinct cohort of targets by a unique mechanism of action. By identifying this transformation specific subset of Myc target genes we aim to develop a diagnostic tool to identify oncogenic Myc *activity* in breast tumor cells. We also aim to develop a unique anti-cancer therapeutic that will potentially target this unique transforming activity of Myc. The TRRAP cofactor has been shown to be essential for Myc to drive transformation. This suggests blocking Myc:TRRAP interaction will inhibit the carcinogenic program directed by oncogenic Myc. By conducting the experiments outlined in this proposal we will test a unique hypothesis and will make significant contributions to the molecular diagnosis and treatment of breast cancer that can be applied to the clinic in a timely manner

Specific Aims:

1) Identify tumor-specific, directly-regulated Myc target genes in transformed HMECs and develop a definitive diagnostic tool to detect oncogenic Myc activity in breast cancer.
2) Isolate small molecular weight inhibitors that can disrupt Myc:TRRAP interaction in vivo and identify Myc:TRRAP co-bound target genes in breast cancer.

Body

With support from the DOD, the research outlined in the original proposal has progressed in a steady and productive manner as expected for this second year of funding. To delineate the accomplishments to date, the tasks outlined in the original Statement of Work of the proposal are itemized below (italics) and a progress report for each task provided.

Statement of Work

Task 1: Identify tumor-specific, directly-regulated Myc target genes in transformed HMECs (months 1-36)

a. *Directly compare fresh and formalin-fixed tissue for efficacy of Chip-on-chip assay using samples, including archived samples, prepared by the Andrulis group (months 1-6)*

   Completed in year 1: We anticipated that archived formalin-fixed tissue could be used for ChIP-on-chip analysis, however, over the course of the first year, learned that formalin fixation conducted in pathology labs is not similar to the formaldehyde fixation used in our research lab as an early step of the ChIP-on-chip procedure. We suspect the issue has more to do with the heterogeneity in the methodology used by different technicians/residents over the years in various pathology labs (formalin formulation, time of fixation, etc). To overcome this problem, we evaluated whether frozen tissue could be used as an alternate source. We are pleased to report that we can achieve robust ChIP-on-chip results using 0.03g of frozen tissue. Importantly, we can move forward with the study, using frozen instead of formalin-fixed primary tissue for our ChIP-on-chip analysis.

b. *Evaluate minimum numbers of cells that is required for quality assured Chip-on-chip of tissue sections, test reproducibility (months 6-12)*

   Completed in years 1 and 2: In year 1 we compared and modified two amplification methods to determine how few cells are required to achieve efficient ChIP-on-chip results without introducing bias due to amplification. In year 2, several additional parameters were evaluated and conditions determined to ensure outstanding reproducibility of this new technology. This data was recently presented at the AACR Annual Mtng and a Cold Spring Harbor Systems Biology Mtng. We are now writing this manuscript for publication.

c. *Conduct the Myc specific Chip-on-chip assay in sixplicate with reverse dye labeling on one sample that has been processed for laser capture microdissection for normal and tumor isogenic matched sample, in duplicate (months 12 –18)* Yet to do*.

d. *Conduct Myc specific Chip-on-chip assay on 2 additional genetically similar patient samples (months 12-18)* Yet to do*

e. *Conduct Myc specific Chip-on-chip on 3 similar samples whose underlying genetic abnormalities are distinct from the first series of 3 (months 18-24)* Yet to do*

f. *Conduct data analysis to evaluate how target genes compare between isogenic normal and tumor matched material and between groups that harbor distinct genetic abnormalities and between all samples to identify a cohort commonly bound and regulated by Myc in tumor but not normal tissue. (months 24 –32)* Yet to do*

*Of the original node-negative tumor bank, only the formalin-fixed tissues had been previously analyzed by expression array analyses. As outlined above, we have learned that this material cannot be processed successfully for ChIP-on-chip analysis. In our research we learned that frozen material serves as a robust source of tissue. Our collaborator, Irene Andrulis, had conducted the complimentary expression array analyses on the formalin-fixed material, but had not yet analyzed the frozen material. This information is critical for our work. Thus, our efforts to process this primary tumor material was delayed, but is now ready to proceed. Once we know which tumors have elevated Myc expression, we can further evaluate whether elevated expression is due to amplification of the c-myc locus using the Taqman and FISH assays we

have developed. The expression array analyses is now complete and we can proceed, as proposed. This will be conducted and completed within the 3 years of the grant, only the timing of the work has been altered from year 2 to year 3 of the grant.

g. *Further evaluate the diagnostic potential of this common cohort and evaluate whether cDNA expression profiling shows these targets are similarly regulated in ANN tumors of a specific subtype or genetic background (months 24 –32)* Yet to do

h. *Extend analysis to tissue arrays using in situ hybridization or IHC (months 24-36)* Yet to do

i. *Evaluate cDNA expression array data for genes identified as regulated by Myc in the MCF10A system (months 12-36)* Yet to do

Task 2: Establish MCF10A cell system and identify tumor-specific, directly-regulated Myc target genes (months 1-36)

a. *Introduce ectopic Myc expression in the MCF10A cells and evaluate biological effect at the level of proliferation, apoptosis induction in standard culture conditions (months 1-6).* Completed in year 1. We ectopically expressed Myc in the MCF10A cells and characterized cell growth and death, as proposed. Myc potentiates both cell proliferation and apoptosis, as expected. Results were presented in last year's annual report.

b. *Conduct Myc ChIP-on-chip analysis and cDNA array analysis to identify target genes directly bound and regulated by Myc under asynchronous conditions (months 1-12).* Completed in years 1 and 2. We assayed non-transformed MCF10A cells, which we had engineered to express control retrovirus or retrovirus expressing ectopic Myc, for their ability to grow in soft agar. Much to our pleasant surprise, deregulated Myc expression was sufficient to enable these cells to grow under anchorage-independent conditions and form colonies in soft agar. Thus, we have developed a model system to identify Myc target genes important for transformation.

Using this system, we conducted ChIP analysis and identified H19 as a novel Myc target gene regulated by Myc in MCF10As. We further showed that Myc induction of H19 plays a role in Myc induced transformation by conducting siRNA knock-downs in breast cancer cell lines that do and do not express H19. Indeed, the loss of H19 inhibits full transformation potential of these breast cancer cells. Thus we show that we have identified at least one novel Myc-induced gene (H19), using the MCF10A system, that plays an important role in transformation. Only a handful of Myc target genes have been shown to play a role in transformation, thus it was important to focus on this single important target and ensure the publication of this information in a timely manner. To this end, a manuscript describing this work has recently been published in *Cancer Research* (Appendix 1). In addition, it will be highlighted in *Nature Reviews Cancer* in the July 2006 issue (Appendix 2). Using using the MCF10A system and our ChIP-on-chip analysis, we aim to identify similar Myc targets that are, like H19, Myc-regulated and key for transformation, as originally proposed.

c. *Into the control and Myc expressing cells introduce activatable erbB2 and p53-DD (months 6-12).* No longer necessary. Originally it remained unclear whether deregulated Myc alone would be able to transform the MCF10A cells, as assessed by anchorage-independent growth in soft agar. It was anticipated that additional genetic

lesions, such as erbB2 and/or p53-DD would be necessary and was proposed. However, these additional genetic lesions are not required. Ectopic Myc alone is able to transform the MCF10A cells. Thus our experimental model system is established in the absence of erbB2 and p53-DD.

d. *Assay cells for growth in soft agar as well as proliferation and apoptosis assays (months 12-18)* Complete. The MCF10A +/- Myc cells have been assayed thoroughly and form the basis of our work. Myc potentiates proliferation, apoptosis and growth in soft agar of these cells. Several strains of MCF10A were evaluated and all behaved similarly with respect to Myc effects on cellular growth, death and tumorigenesis.
e. *Assay Myc ChIP-on-chip and cDNA arrays on these cells expressing ectopic Myc and/or erbB2 and/or p53-DD, under asynchronously growing conditions (months 18-32)* Completed and yet to do. The ChIP-on-chip analyses are complete and the expression arrays are now in progress.
f. *Assay more transformed cells for invasion, polarity, morphology properties (months 18 – 32)* Yet to do.
g. *Test for genetic abnormalities and their effects on cell differentiation in matrigel (months 24-36)* Yet to do.
h. *Assay all cells for Myc Chip-on-chip and cDNA arrays when grown in soft agar (months 24-36)* Yet to do.

Task 3:Isolate small molecular weight inhibitors that can disrupt Myc:TRRAP interaction in vivo and identify Myc:TRRAP co-bound target genes in breast cancer.

a. *Develop and test antibodies to TRRAP for ChIP (months 1-12)* Completed. After much trouble-shooting we are able to readily ChIP TRRAP from live cells using a homemade antibody. The specificity of the antibody has been rigorously evaluated and the ChIP results are robust and quantitative. This will enable TRRAP ChIP-on-chip to be conducted in the MCF10A cell system described above. We aim to also advance these assays to primary patient material, as proposed. By this approach the target genes that are co-bound by Myc and TRRAP, that are essential for transformation, will be identified.
b. *Develop Myc and TRRAP interacting fragments in new screening system and evaluate interaction and susceptibility to inhibition with TRRAP polypeptide (months 1-12)* Complete and in progress. Before setting up the screening system we needed to ensure the fragment of TRRAP shown to interact with Myc did indeed interact with Myc in vivo. This has been evaluated and is about to be submitted for publication (Appendix 3). The amino acid details have been removed from this document, for the purposes of confidentiality while the paper is under review for patent potential. I hope you understand. Targeting Myc as a novel anti-cancer therapeutic has enormous potential (for more information, see our review Appendix 4). With these results we went forward to establish the RTA screening system, as proposed, but the system did not work with our positive controls. The RTA was modified accordingly so that inhibitors show a robust regulation of indicator gene expression, so the yeast system can now be used for the screen. This is ongoing now.

c. *Conduct screen and test positives in the RTA against Myc:TRRAP as well as other interactors of Myc and other interactors of TRRAP to evaluate specificity of the inhibitor (months 12-18)* In progress.

d. *Advance inhibitors to mammalian cell assays, including growth, transformation DNA binding using a Chip-on-chip approach (months 18-36)* Yet to do.

Key Research Accomplishments

- We have identified H19 as a bone fide Myc target gene that contributes to transformation of breast cancer. This was recently published.
- We have established the deregulated Myc expression is sufficient to transform MCF10A cells as defined by anchorage independent growth in soft agar. This system is ideal to further identify the entire subset of genes Myc regulated to transform human mammary epithelial cells. Indeed, it was this system that enabled the identification of H19 as our prototypic gene with these critical characteristics.
- Myc and TRRAP interaction domains have been thoroughly analyzed and fully characterized. This has resulted in a publication that is nearly ready for submission.
- We have re-tooled the RTA so that the indicators are more robust for the proposed screen. We switched to a his-based indicator system using growth in the absence of histidine as our selection platform.

Reportable Outcomes

- Barsyte-Lovejoy D, Lau SK, Boutros PC, Khosravi F, Jurisica I, Andrulis IL, Tsao MS, Penn LZ. The c-Myc oncogene directly induces the H19 noncoding RNA by allele-specific binding to potentiate tumorigenesis. Cancer Res. 2006 May 15;66(10):5330-7. (see Appendix 1)
- Nature Reviews Cancer – The Silent Messenger - highlight of Cancer Research article above (see Appendix 2)
- Ponzielli R, Katz S, Barsyte-Lovejoy D, Penn LZ. Cancer therapeutics: targeting the dark side of Myc. Eur J Cancer. 2005 Nov;41(16):2485-501. Review. (see Appendix 4)
- Lilia Kaustov, Sigal Katz, Cynthia S.W. Ho, Romina Ponzielli, Shili Duan, Steve McMahon, Michael D.Cole, Linda Z. Penn, and Cheryl H. Arrowsmith , Characterizing cMyc MBII and its interaction with TRRAP, soon to be submitted, (see Appendix 3)
- Optimizing ChIP-on-chip and reporting on key parameters of technology – presented at AACR Annual Meeting, Washington, DC, April 1-5, 2006 (Penn oral presentation, Boutros poster presentation); also presented at Cold Spring Harbor Labs, System Biology Conference, Cold Spring Harbor, NY, Mar 23 –26, 2006 (Boutros poster presentation)
- Oral presentation by Penn at "Reasons for Hope" Conference, sponsored by the Canadian Breast Cancer Research Alliance, Montreal, Quebec, May 6-8, 2006

Conclusions

The work to date shows the ultimate goals of the proposal will be accomplished during the tenure of this award. Several publications have arisen from this research and several additional significant contributions are expected. In the second year of funding we built on the strong foundation of the first year and made progress in several areas:

1) We established a useful model system to recapitulate the transition from non-transformed (MCF10A cells) to transformed (MCF10A cells with deregulated Myc expression) human mammary epithelial cells. This system is the best we could have hoped for. It is

simple and does not involve the need to introduce additional genetic abnormalities to achieve anchorage independent growth. This enables the genetics to be readily interpreted. Indeed, this system enabled the Myc target gene H19 to be identified and quickly evaluated for it mechanism of regulation and importantly, its role in breast cancer transformation.

2) We have developed the technologies in the ChIP-on-chip system to move forward relatively quickly to evaluate the frozen primary breast cancer tissue, as originally proposed. We are now analyzing the expression array data to determine which tumors to further evaluate by Taqman, FISH and ChIP-on-chip.
3) We have developed critical antibodies that recognize TRRAP and work in ChIP to identify TRRAP-bound target genes in a sensitive and specific manner. These valuable reagents will be used for further ChIP-on-chip analyses, as proposed.
4) We have established the regions of Myc and TRRAP to use in the RTA screen which we have recently modified for use in the high throughput screen for inhibitors that block this key interaction essential for Myc-induced transformation.

Thus, both Aims 1 and 2 are proceeding well and will be achieved within the time frame of this grant proposal.

References

1. S. Oster, C. Ho, E. Soucie, L.Z. Penn**,** *Advances in Cancer Research*. **84, 81** (2002).

2. R. Ponzielli, S. Katz, D. Barsyte-Lovejoy, L.Z. Penn, *Eur J of Cancer*. **41, 2485** (November 2005)

Appendices

Appendix 1: Barsyte-Lovejoy D, Lau SK, Boutros PC, Khosravi F, Jurisica I, Andrulis IL, Tsao MS, Penn LZ. The c-Myc oncogene directly induces the H19 noncoding RNA by allele-specific binding to potentiate tumorigenesis. Cancer Res. 2006 May 15;66(10):5330-7.

Appendix 2: Nature Reviews Cancer – The Silent Messenger - highlight of Cancer Research article above

Appendix 3: Lilia Kaustov, Sigal Katz, Cynthia S.W. Ho, Romina Ponzielli, Shili Duan, Steve McMahon, Michael D.Cole, Linda Z. Penn, and Cheryl H. Arrowsmith , Characterizing cMyc MBII and its interaction with TRRAP, soon to be submitted

Appendix 4: Ponzielli R, Katz S, Barsyte-Lovejoy D, Penn LZ. Cancer therapeutics: targeting the dark side of Myc. Eur J Cancer. 2005 Nov;41(16):2485-501. Review.

The c-Myc Oncogene Directly Induces the H19 Noncoding RNA by Allele-Specific Binding to Potentiate Tumorigenesis

Dalia Barsyte-Lovejoy,[1] Suzanne K. Lau,[3,4] Paul C. Boutros,[1,2,4] Fereshteh Khosravi,[1] Igor Jurisica,[2,4,6] Irene L. Andrulis,[7] Ming S. Tsao,[3,4,5] and Linda Z. Penn[1,4]

Divisions of [1]Cancer Genomics and Proteomics, [2]Signaling Biology, and [3]Applied Molecular Oncology, Ontario Cancer Institute/Princess Margaret Hospital; Departments of [4]Medical Biophysics and [5]Laboratory Medicine and Pathobiology, [6]Computer Science, University of Toronto, Canada; and [7]Fred Litwin Cancer Genetics Center, Samuel Lunenfeld Research Institute Mount Sinai Hospital, Toronto, Ontario, Canada

Abstract

The product of the *MYC* oncogene is widely deregulated in cancer and functions as a regulator of gene transcription. Despite an extensive profile of regulated genes, the transcriptional targets of c-Myc essential for transformation remain unclear. In this study, we show that c-Myc significantly induces the expression of the *H19* noncoding RNA in diverse cell types, including breast epithelial, glioblastoma, and fibroblast cells. c-Myc binds to evolutionarily conserved E-boxes near the imprinting control region to facilitate histone acetylation and transcriptional initiation of the *H19* promoter. In addition, c-Myc down-regulates the expression of insulin-like growth factor 2 (*IGF2*), the reciprocally imprinted gene at the *H19/IGF2* locus. We show that c-Myc regulates these two genes independently and does not affect *H19* imprinting. Indeed, allele-specific chromatin immunoprecipitation and expression analyses indicate that c-Myc binds and drives the expression of only the maternal *H19* allele. The role of H19 in transformation is addressed using a knockdown approach and shows that down-regulation of H19 significantly decreases breast and lung cancer cell clonogenicity and anchorage-independent growth. In addition, c-Myc and H19 expression shows strong association in primary breast and lung carcinomas. This work indicates that c-Myc induction of the *H19* gene product holds an important role in transformation. (Cancer Res 2006; 66(10): 5330-7)

Introduction

The transforming members of the Myc family (c-Myc, N-Myc, and L-Myc) show deregulated expression in a broad spectrum of cancers, including carcinomas of the lung, breast, and prostate as well as leukemias and lymphomas (1). c-Myc is a transcription factor that, with its obligate heterodimerization partner Max, binds to DNA sequence elements called E-boxes (2). c-Myc-Max can subsequently recruit histone acetyltransferase (HAT) activity (3), chromatin remodeling complexes (4), or promote RNA polymerase II (RNAPII) clearance (5) to allow for target gene transcription. c-Myc-Max can also repress gene transcription primarily by interfering with the assembly or function of the transcriptional complex (6–8). As a transcription factor, c-Myc regulates numerous gene targets that subsequently execute its many biological activities, including cell proliferation, transformation, angiogenesis, and apoptosis (9). Identifying these target genes is key in elucidating the role of this potent oncogene in transformation and has thus received much attention. Despite an extensive list of c-Myc-regulated genes, it remains unclear which cohort of target genes is responsible for the strong transforming activity of c-Myc (10, 11).

Recent analyses using advanced high-throughput chromatin immunoprecipitation technology has revealed the nature of the target genes whose promoter regulatory regions are bound by c-Myc (12–15). Remarkably, these analyses have indicated that c-Myc target genes include both coding and noncoding RNAs (ncRNA; ref. 16). Noncoding RNAs are transcripts expressed and processed in the nucleus in a manner similar to protein coding genes; however, ncRNAs lack a conserved open reading frame. Although elucidating the function of ncRNAs is in the early stages of investigation, evidence suggests that at least some may have roles in tumorigenesis (17). To determine the role of c-Myc-regulated ncRNAs in transformation, we investigated both the regulation and function of the large prototypic ncRNA, H19, as a downstream target of c-Myc.

H19 was first described as a tumor suppressor (18, 19), but more recent analysis shows that H19 expression is reactivated in breast (20), endometrial (21), lung (22), cervical (23), esophageal (24), and bladder (25) tumors. The *H19*/insulin-like growth factor 2 (*IGF2*) locus, containing both the *H19* and *IGF2* genes, is subject to genomic imprinting, which leads to differential allelic expression of H19 from the maternal allele and IGF2 from the paternal allele (26). This allele-specific expression is highly regulated by differential methylation of CpG dinucleotides that are usually concentrated in CpG islands, genomic elements that are often located close to promoter regions (27). As our data indicated that c-Myc can bind to intergenic regions containing CpG islands (13), we also explored the consequences of allele-specific CpG methylation on the transcriptional regulatory function of c-Myc at the *H19/IGF2* locus.

In this study, we show that c-Myc induces the expression of the H19 ncRNA and binds directly to E-boxes close to the imprinting control region (ICR). Using allele-specific chromatin immunoprecipitation analysis, we show that c-Myc specifically binds and regulates the active maternal *H19* allele and does not bind or affect the expression of the silenced paternal allele. In addition, c-Myc down-regulates transcription of the reciprocally imprinted gene *IGF2*. The significance of H19 up-regulation by c-Myc and the association of c-Myc and H19 transcript levels were assessed in primary and established tumor cells derived from breast and lung cancer patients.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Linda Z. Penn, Division of Cancer Genomics and Proteomics, Ontario Cancer Institute/Princess Margaret Hospital, 610 University Avenue, Toronto, Canada M5G 2M9. Phone: 416-946-2276; Fax: 416-946-2840; E-mail: lpenn@uhnres.utoronto.ca.

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Appendix 1

Materials and Methods

Cell lines. The immortal, nontransformed MCF10A breast cell line (gift from Dr. Muthuswamy) was cultured as described (28). Cells were grown with 10% fetal bovine serum in α-MEM (glioblastoma T98G), DMEM H21 (rat cardiomyocyte H9C2 and fibroblast Rat1MycER^{TAM}), McCoy's (breast cancer cell lines MDA-MB231, SKBR3, and colon carcinoma line HCT116), and RPMI 1640 (T47D breast cancer cell line and lung cancer cell lines A549, H460, and H520). Rat Myc null cells (HO15.19) were grown in DMEM H21 with 10% calf serum. Where indicated, 5 μmol/L 5-aza-deoxycytidine (AzaC; Sigma, St. Louis, MO) was added to cells every 24 hours. Trichostatin A (TSA; Calbiochem, La Jolla, CA) was used at 300 nmol/L.

Retroviral gene transfer. Ectopic human c-Myc was introduced by infection with ecotropic, replication-incompetent retrovirus, and expression was confirmed as described (29).

Isolation and analysis of RNA. Total RNA was isolated as previously described (30) and purified using the RNeasy Mini kit (Qiagen, Inc., Chatsworth, CA). Five micrograms of total cellular RNA were reverse transcribed using Superscript II Reverse Transcription reagents and OligoD_T (Invitrogen, San Diego, CA). Northern blots were done as described (11).

Gene expression analysis in cancer cell lines. Semiquantitative reverse transcription-PCR (RT-PCR) was carried out as described (13) with primers provided in Supplementary Table S1, and the conditions are available upon request. For the quantitative real-time RT-PCR analysis of the breast and lung cell lines, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) were used according to the manufacturer's instructions. A total of one thousandth of the cDNA reaction was used for each PCR triplicate. The results were normalized to the levels of the 36B4 transcript using the comparative C_t method. The allele-specific PCR employed the forward primers specific for the particular *H19* allele, CGGCTCTCGAAGGTGAAGCT (B) or CGGCTCTCGAAGGTGAAGCG (A), and the reverse primer used was TCGTGGAGGCTTTGAATCTCTCAG.

Selection of patients. The RNA expression profiles of a total of 186 breast cancer samples, representing 137 distinct tumors from the cohort described (31), were assayed using cDNA expression microarrays. Two samples lacked follow-up data and were excluded from further analysis. A total of 240 snap-frozen non–small cell lung (NSCLC) carcinoma samples were harvested from patients who have been treated primarily by surgical resection at the University Health Network from 1996 to 2000. Tissues were banked with informed consent, and the studies have been approved by the institutional Research Ethics Board.

Analysis of breast tumor expression data. Raw microarray images were quantitated with the GenePix (Axon, Union City, CA) software package with flagging of low-quality spots. Spot signal was calculated by subtracting the median background pixel intensity from the mean foreground pixel intensity. Ratios were transformed into $\log_2$ space, and missing values were imputed from spotwise duplicates where possible. Data were normalized by sequential print-tip loess smoothing (within array) and scale adjustment (between arrays) as described by Yang et al. (32). All normalization employed the limma package of the Bioconductor library for R (v 2.0.1; ref. 33). Normalized expression values were centered using a 15% trimmed mean. To assess the spot quality, we determined the correlation of the two sequences representing H19 (BI092679 and BQ028553) and MYC (H43827 and W87741). Five outliers with extreme *H19* expression were identified via the *Q* test and removed, leading to higher spot correlation ($R = 0.72$). Spots were then collapsed by averaging. Similarly, the expression from two MYC spots were found to be largely uncorrelated ($R = -0.07$); thus, the clone most specific to MYC was selected. These normalized data represented 137 distinct tumor samples, with clinical follow-up available for 135 of these. These samples were dichotomized around the median H19 expression level. A *t* test with the assumption of unequal variances was used to test MYC expression for significant differences. Normalized array data for all spots are available as Supplementary Table S2.

Quantification and analysis of *MYC* and *H19* expression in lung tumor samples. Real-time quantitative PCR amplification was conducted using the SYBR Green assay in the ABI PRISM 7900-HT (Applied Biosystems). Each 10-μL quantitative RT-PCR reaction contained a 2-ng equivalent of cDNA in a 384-well plate. The reactions were activated at 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 65°C for 15 seconds, and 72°C for 20 seconds. The transcript number/ng cDNA was obtained using standard curves generated with a pool of 10 nontumor lung genomic DNAs. Primer sequences are provided in Supplementary Table S1. Duplicate RT samples were used in each assay. Technical replicates displayed high correlation ($R_{avg} = 0.96 \pm 0.04$; Supplementary Fig. S1) and were then collapsed through averaging. First, expression values were $\log_2$ transformed after addition of a pseudocount. Samples lacking H19 or TBP signal were removed from the data set. For each of the remaining samples, a normalization factor was calculated using the mean of the four housekeeping genes (*TBP, ACTB, B2M*, and *BAT1*) and used to remove nonbiologicalvariability.Forthe*i*thpatient,thenormalizationfactoris N_i $N_i = 0.25 \times [X_i(\text{TBP}) + X_i(\text{ACTB}) + X_i(\text{B2M}) + X_i(\text{BAT1})]$, and normalized expression values for gene M are $Y_i(M) = X_i(M) - N_i$, where the X_i values correspond to unnormalized expression values. For each patient cohort, the normalized expression values were median centered to yield the final expression estimates: $Z_i(M) = Y_i(M) - \text{median}[Y(M)]$. The two patient cohorts were then merged, and the overall data set of 240 distinct samples was dichotomized around the median H19 expression level. A *t* test with the assumption of unequal variances was used to test for differential c-Myc expression. Raw quantitative RT-PCR data are available online as Supplementary Table S3.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done as previously described (13) using the following antibodies: 2 μg Myc (Sc-764), 1 μg CTCF, 0.5 μg RNAPII (Sc-764), 1 μg Max, all from Santa Cruz Biotechnology (Santa Cruz, CA), and 0.5 μg of AcH3 or AcH4 (Upstate Biotechnology, Lake Placid, NY). Real-time PCR was done as described above using human genomic DNA as the standard and normalizing the specific antibody signal to the input signal. Primer sequences are provided in the Supplementary Table S1, and the conditions are available upon request. The same chromatin immunoprecipitation material was used for allele specific PCR with allele-specific primers CGCCTACTTATGTGATGATCAG or CGCCTACTTATCTGATGATCAC and the reverse GCACCCACGATAATGGATT.

H19 knockdown. The small interfering RNAs (siRNA) were designed using the SFold web site (http://sfold.wadsworth.org). The control siRNA was against luciferase (34), whereas H19 hairpin oligo sequences are CCGGGCGGGTCTGTTTCTTTACTTTCAAGAGAAGTAAAGAAACAGACC-CGCTTTTTG and reverse AATTCAAAAAGCGGGTCTGTTTCTTTACTTCT-CTTGAAGTAAAGAAACAGACCCGC. The annealed phosphorylated oligos were cloned into pLKO1puro lentiviral vector (gift from Drs. Stewart, Novina, and Weinberg). These constructs, together with packaging vectors pMD.G, pMDLg/pRRE and pRsv-Rev (gift from Dr. Naldini), were transfected into 293TV cells, and viral supernatant was collected 48 hours later and used to infect the cells.

Anchorage and clonogenicity assays. Anchorage-independent growth assays were done as described (29), except 5,000 cells were seeded and counted at the end of a 2-week period. For clonogenicity assays, 500 cells were seeded in six-well dishes, and the media containing puromycin was changed every 3 days. After the 1- to 2-week period, the resulting colonies were stained with 2% methylene blue in 50% ethanol and counted.

Results

c-Myc up-regulates H19 and down-regulates IGF2 transcripts. The *H19/IGF2* locus is subject to genomic imprinting (Fig. 1*A*). Allele-specific methylation of CpG dinucleotides in the ICR leads to H19 expression from the maternal allele, whereas the reciprocally imprinted *IGF2* gene is expressed from the paternal allele (26). The ICR on the maternal allele is unmethylated and bound by CTCF, a zinc-finger protein that acts as a boundary between the enhancers located 3′ of H19 and the promoters of IGF2. The paternal allele is methylated at the ICR, preventing

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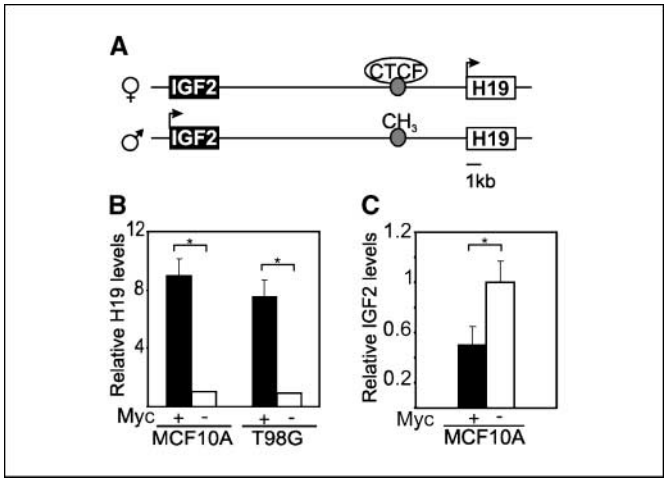

Figure 1. Ectopic Myc induces the expression of H19 and represses IGF2 expression. *A,* schematic of the *H19/IGF2* locus, where active promoters are denoted with arrows and the imprinting control region (*gray circles*) is shown bound by CTCF or methylated (CH_3). *B,* H19 ncRNA expression in MCF10A and T98G cells with (+) and without (−) ectopic Myc as assessed by quantitative RT-PCR. 36B4 ribosomal protein mRNA was used as a normalization control. *C,* IGF2 mRNA levels in MCF10A cells. Quantitative RT-PCR was conducted in triplicates twice. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistical significance as assessed using a paired *t* test.

CTCF binding, allowing the enhancers to potentiate IGF2 transcription (26).

To evaluate whether c-Myc regulates *H19* expression, we introduced ectopic c-Myc into several cell types, including MCF10A immortalized nontransformed mammary epithelial cells and T98G glioblastoma cells (Supplementary Fig. S2A). Cells with ectopic c-Myc expression showed 7- to 10-fold up-regulation of H19 ncRNA expression as assessed by real-time quantitative RT-PCR (Fig. 1*B*), semiquantitative RT-PCR, and Northern blotting (Supplementary Fig. S2B). This up-regulation was also evident in diploid fibroblasts WI38 (data not shown) and a medulloblastoma cell line UW228 (see Fig. 3*B*). The activation of the constitutively expressed c-Myc/estrogen receptor regulatory region ($MycER^{TAM}$) chimera by 4-hydroxytamoxifen also resulted in elevated H19 expression in Rat-1 fibroblasts (Supplementary Fig. S2C).

Given the strong up-regulation of H19, we assessed the expression of IGF2 in response to exogenous c-Myc expression. Interestingly, c-Myc down-regulated IGF2 in several cell systems, such as MCF10A and WI38 (Fig. 1*C*; data not shown). However, in T98G cells, which have low to undetectable levels of IGF2, down-regulation of IGF2 was not consistently detectable (data not shown). This prompted further evaluation in rat cardiomyocytes that have high basal levels of IGF2 transcripts. c-Myc robustly repressed the levels of IGF2 in H9C2 cardiomyocytes as shown by Northern blot (Supplementary Fig. S2D). Thus, c-Myc strongly up-regulates H19 and down-regulates IGF2 transcript levels in several cell types.

c-Myc directly binds to the regulatory regions of *H19* and *IGF2*. To determine whether c-Myc directly regulates H19, we assessed *in vivo* genomic DNA binding of c-Myc to the regulatory region of H19 using chromatin immunoprecipitation, focusing on the evolutionary conserved E-boxes situated 1.5 and 3.1 kb upstream from the transcription start site. c-Myc immunoprecipitates were highly enriched in these DNA fragments compared with control serum immunoprecipitates (Fig. 2*A*, primer sets 1 and 3). The ICR region containing the second cluster of three DNase hypersensitive sites essential for CTCF binding showed weaker binding of c-Myc (Fig. 2*A*, primer set 2). Interestingly, the abovementioned E-boxes are 650 and 360 bp away from the second hypersensitive site cluster within the ICR, and there are also numerous noncanonical E-boxes interspersed between the

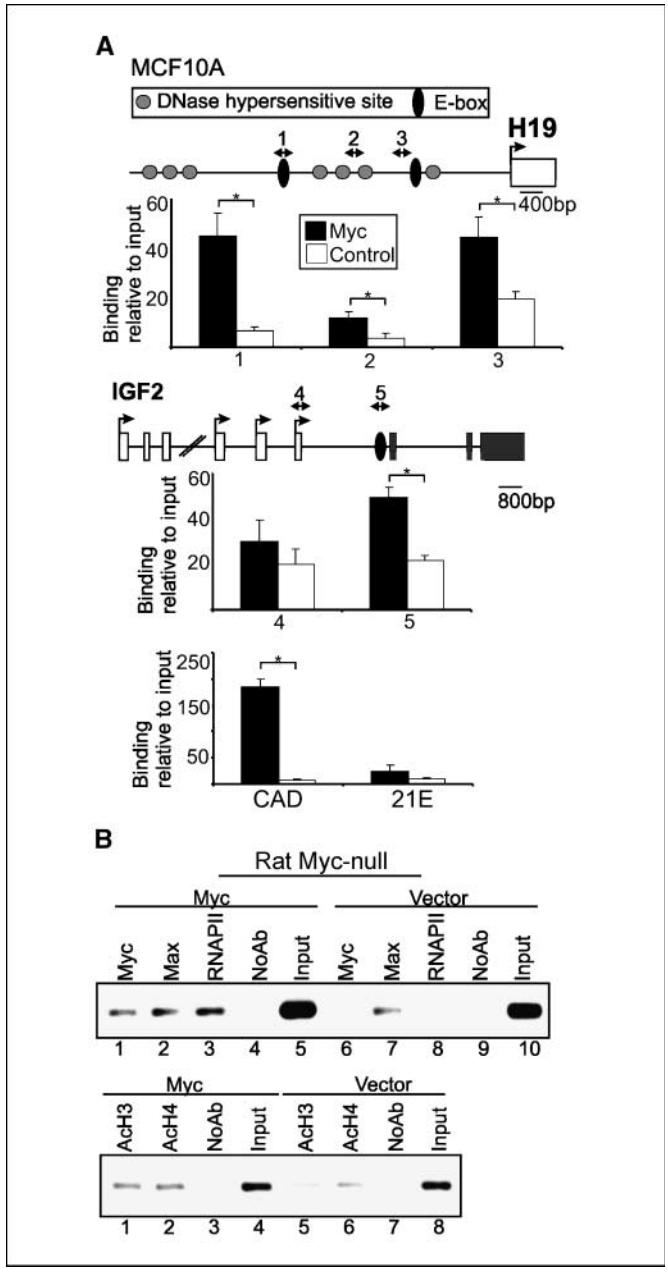

Figure 2. Myc binds to the promoter regions of H19 and IGF2 to regulate gene transcription. *A,* Myc binding at the regulatory region of the *H19* and *IGF2* genes, as well as the CAD (positive control) and chromosome 21 E-box (negative control) was assessed by chromatin immunoprecipitation using Myc-specific antibody (*Myc*) or preimmune serum (*Control*) in MCF10A cells expressing ectopic Myc. Primers are indicated by arrows. DNase-hypersensitive sites (*gray circles*) comprise the ICR. Relative binding was normalized to the input signal and expressed as 0.001% of input. Quantitative PCR was conducted in triplicates twice. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistical significance as assessed using a paired *t* test. *B,* the transcriptional components bound at the *H19* promoter were assessed by chromatin immunoprecipitation using primers to the proximal E-box (equivalent to primer set 3 in *A*). Rat Myc-null cells that were reconstituted with Myc (*Myc*) or control green fluorescent protein vector (*vector*) were assayed using indicated antibodies. Chromatin immunoprecipitation experiments were repeated at least twice with representative gels shown.

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hypersensitive sites. Therefore, from the binding data alone it remained unclear whether Myc would regulate the promoter of H19 or the ICR and imprinting. This issue is addressed through functional analysis below. We have also investigated c-Myc binding to the *IGF2* gene using a scanning chromatin immunoprecipitation approach. *IGF2* is expressed from the promoter P4 in MCF10A cells (data not shown); however, c-Myc binding was weak or undetectable not only at the P4 promoter but also at several other sites, including the differentially methylated region 1 and the coding region in the exon 9 (Fig. 2*A*, primer set 4; data not shown). Only the E-box 5′ from coding exon 7 showed binding of c-Myc (Fig. 2*A*, primer set 5). The positive control *CAD* promoter was highly enriched in c-Myc immunoprecipitates, whereas the negative control E-box at chromosome 21 showed no significant enrichment (Fig. 2*A*). Thus, c-Myc binds to the regulatory regions of *H19* and *IGF2 in vivo*.

c-Myc recruits HATs to the *H19* promoter. To investigate evolutionary conservation and further assess the mechanism of *H19* transcriptional regulation by c-Myc, we compared rat fibroblast cells that are devoid of Myc expression with those that have been reconstituted with ectopic c-Myc for promoter occupancy by chromatin immunoprecipitation analysis. In the absence of c-Myc, Max is bound to the *H19* promoter proximal E-box, a feature previously described as a hallmark of c-Myc-regulated genes (Fig. 2*B, top, lane 7*; ref. 13). Reintroducing c-Myc into these cells resulted in binding of c-Myc, the recruitment of RNAPII (Fig. 2*B, top, lanes 1* and *3*) and an increase in the acetylation of histone H3 (AcH3) and H4 (AcH4) at the *H19* E-box (Fig. 2*B, bottom, lanes 1* and *2*). The role of histone acetylation in c-Myc-mediated transcriptional induction of *H19* was further supported by evaluating the effect of the histone deacetylase (HDAC) inhibitor TSA. Treatment with TSA resulted in increased expression of H19 in $myc^{-/-}$ cells as detected by semiquantitative RT-PCR (Fig. 3*A, lanes 1-4*). Curiously, the level of *H19* expression achieved by TSA treatment was higher in $myc^{-/-}$ cells than in c-Myc-expressing cells (Fig. 3*A, lanes 3* and *4*). Thus, histone acetylation activity is recruited by c-Myc to the *H19* promoter, leading to its activation.

c-Myc does not affect imprinting of the *H19/IGF2* locus. Because the paternal allele of *H19* is usually silent due to ICR methylation (see Fig. 1*A*), the effect of DNA methylation on c-Myc regulation of H19 and IGF2 was further investigated. *H19* expression was evaluated in $myc^{-/-}$ cells and c-Myc reconstituted cells in the presence and absence of DNA methylation inhibitor AzaC. Exposure to AzaC did not result in H19 up-regulation in $myc^{-/-}$ cells (Fig. 3*A*, compare *lanes 1, 5*, and *7*), whereas blocking DNA methylation increased *H19* expression in c-Myc-reconstituted cells (Fig. 3*A*, compare *lane 2* with *lanes 6* and *8*). By removing DNA methylation (AzaC) and then increasing histone acetylation (TSA), *H19* expression was potentiated in $myc^{-/-}$ cells (Fig. 3*A*, compare *lanes 1, 3*, and *9*), and no further induction by c-Myc was evident under these conditions (Fig. 3*A, lanes 9* and *10*). Blocking DNA methylation also did not affect IGF2 basal gene expression (Fig. 3*A*, compare *lanes 1, 5*, and *7*) but further potentiated c-Myc repression of this target gene (Fig. 3*A*, compare *lanes 2, 6*, and *8*). c-Myc repression of *IGF2* remains intact despite treatment with both TSA and AzaC (Fig. 3*A, lanes 9* and *10*). These data suggest that c-Myc regulation of the *H19/IGF2* locus does not involve DNA methylation or imprinting. Moreover, loss of methylation alone is insufficient to alter basal gene expression but can cooperate with c-Myc to potentiate the regulation of these target genes.

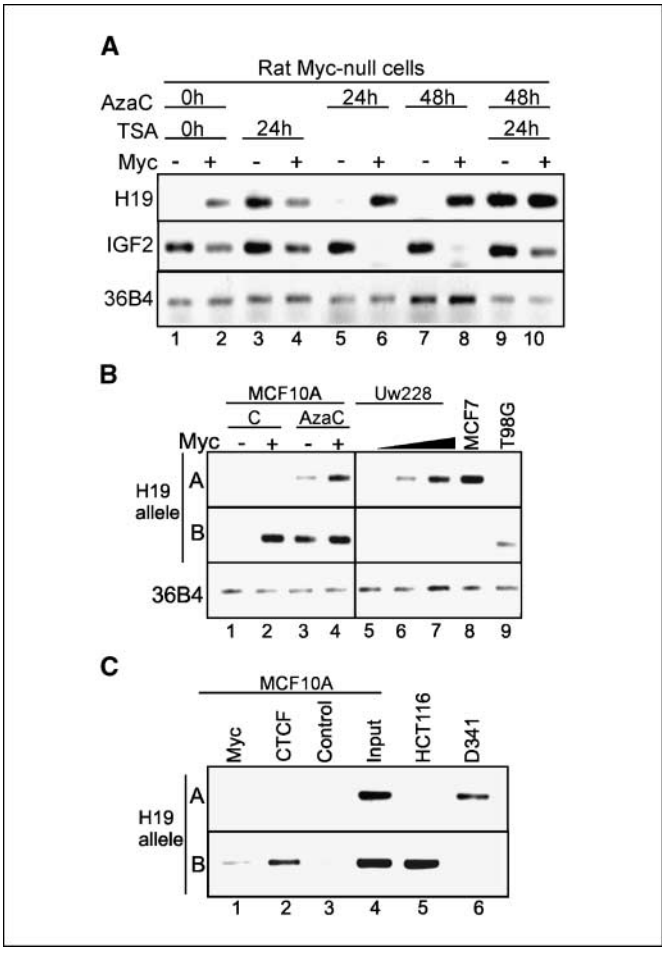

Figure 3. Myc binds and regulates only one allele of H19. *A,* semiquantitative RT-PCR of H19 and IGF2 in Myc null rat fibroblasts expressing the control vector (−) or ectopic Myc (+) that were exposed to HDAC inhibitor TSA and/or DNA methylation inhibitor AzaC for the time indicated. The ribosomal protein 36B4 mRNA serves as a control. Semiquantitative RT-PCR was conducted twice with similar results. *B,* allelic expression analysis of H19 using semiquantitative RT-PCR. *C,* allele-specific binding by Myc and CTCF in MCF10A cells that were used for chromatin immunoprecipitation with Myc-specific and CTCF-specific antibodies, and the immunoprecipitates were analyzed with allele-specific primers. PCR analysis was conducted thrice; representative data are shown.

c-Myc up-regulates only one allele of *H19*. Because the *H19/IGF2* locus is subject to genomic imprinting, we had a unique opportunity to investigate the transcriptional regulatory effect of c-Myc on the allelic expression of *H19*. Multiple cell lines were first tested for polymorphisms in the *H19* locus (data not shown), and the allele-specific primers A and B (*Alu*I polymorphism) were then designed, differing by a 3′ single nucleotide. The primers were tested on the homozygous MCF7 and T98G cells to show allelic specificity (Fig. 3*B, lanes 8* and *9*). In MCF10A cells, ectopic expression of c-Myc induced expression of *H19* from the single allele B (Fig. 3*B, lanes 1* and *2*). c-Myc induction of both alleles of H19 was evident in the presence of AzaC, showing that in the absence of DNA methylation, c-Myc can access and activate both alleles of *H19* (Fig. 3*B, lanes 3* and *4*). Similarly, ectopic expression of increasing amounts of c-Myc activates only the single allele A of *H19* in a dose-dependent manner in Uw228 medulloblastoma cells (Fig. 3*B, lanes 5-7*). Therefore, c-Myc induces *H19* expression by a mechanism that is restricted to only one allele of *H19*.

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c-Myc binds to the promoter of one *H19* allele. To further evaluate the molecular mechanism of c-Myc induced allele-specific expression of *H19,* we queried whether c-Myc binds to only one or both alleles of *H19 in vivo*. Allele-specific chromatin immunoprecipitation primers were tested on control DNA from homozygous HCT116 and D341 cell lines to ensure that only one allele is recognized using this approach (Fig. 3*C, lanes 5* and *6*). Both c-Myc and CTCF are bound to allele B but not allele A in MCF10A cells (Fig. 3*C*, compare *lanes 1-3* and *lanes 2-3*). The weaker signal for c-Myc binding could be due to the allele-specific primers being situated 0.4 kb away from the E-box. The bound allele was designated as allele B (Fig. 3*B*) because it has been reported that CTCF binds to the maternally derived, expressed allele of *H19* (26). Indeed, allele B of *H19* is bound by c-Myc and CTCF (Fig. 3*C, lanes 1* and *2*), whereas allele A is not bound by either c-Myc or CTCF, nor it is sensitive to induced expression in response to ectopic c-Myc in MCF10A cells. These allelic binding results were also confirmed by methyl-specific PCR (data not shown). Thus, c-Myc and CTCF bind to the maternally derived, expressed allele of *H19*.

H19 knockdown inhibits tumorigenic properties of breast and lung cancer cells. To assess the role of H19 in transformation, we evaluated the expression and functional significance of H19 in cells derived from breast and lung carcinomas. H19 has recently been reported to be elevated in a high proportion of these tumor types (20, 22). H19 expression was evident in the SKBR3 and T47D breast cancer cell lines and the lung adenocarcinoma A549 cells, whereas expression was undetectable in MDA-MB231 breast cancer cell line (Fig. 4*A*). Stable siRNA-mediated knockdown of H19 resulted in significant decrease in both clonogenicity, as assessed by the efficacy of colony formation on solid support (Fig. 4*B*), and anchorage-independent growth, as assessed by colony formation in soft agar (Fig. 4*C*). The inhibition of both clonogenicity- and anchorage-independent growth in response to H19 knockdown was significant in all cell lines with H19 expression (SKBR3, T47D, and A549), and insignificant in cells with undetectable basal H19 expression, such as MDA-MB231 cells or H460 and H520 lung carcinoma cell lines (Fig. 4*B* and *C*; data not shown). A lack of biological effect of H19 siRNA in MDA-MB231, H460, and H520 serves as an important specificity control. Three of five siRNAs designed worked well to down-regulate H19 expression; however, only one was specific (data not shown). Another siRNA we identified as a nonspecific H19 siRNA was previously used to transiently down-regulate H19 (35). Thus, H19 contributes to the clonogenic and anchorage-independent growth properties in breast and lung cancer cells with reactivated H19 expression.

H19 regulation of IGF2. As H19 has been reported to repress the levels of IGF2, we also monitored the effect of H19 knockdown on IGF2 levels. No significant and consistent increase in IGF2 expression upon H19 repression was noted in any of the breast cancer cells (Fig. 4*D*) or MCF10A cells expressing ectopic c-Myc and elevated H19 (data not shown). Only the A549 cells with H19 knockdown displayed increased IGF2 transcript expression (Fig. 4*D*).

c-Myc binds to the *H19* promoter in SKBR3 but not MDA-MB231 cells. To further evaluate the mechanism of c-Myc induction of *H19* transcription, we queried whether c-Myc was bound to the *H19* E-box (−1.5 kb) in cells with (SKBR3) and without (MDA-MB231) basal *H19* expression. Quantitative chromatin immunoprecipitation analysis showed that c-Myc, CTCF, RNAPII, and AcH4 were present at the *H19* E-box in SKBR3 compared with control antibody (Fig. 5*A*). However, in MDA-MB231 cells, binding of c-Myc and RNAPII was not evident, whereas the binding of CTCF was reduced, and AcH4 was unaffected (Fig. 5*A*). The positive control E-box (CAD) bound c-Myc and RNAPII in both cell lines, whereas the negative control E-box on chromosome 21 had no significant binding of any of the factors (Fig. 5*B* and *C*). Therefore, c-Myc binds to the promoter region of *H19* in SKBR3 cells and contributes to the up-regulation of H19.

High expression levels of H19 correlate with elevated levels of c-Myc mRNA in breast and lung cancer patients. To determine if c-Myc regulation of H19 is a feature of primary human

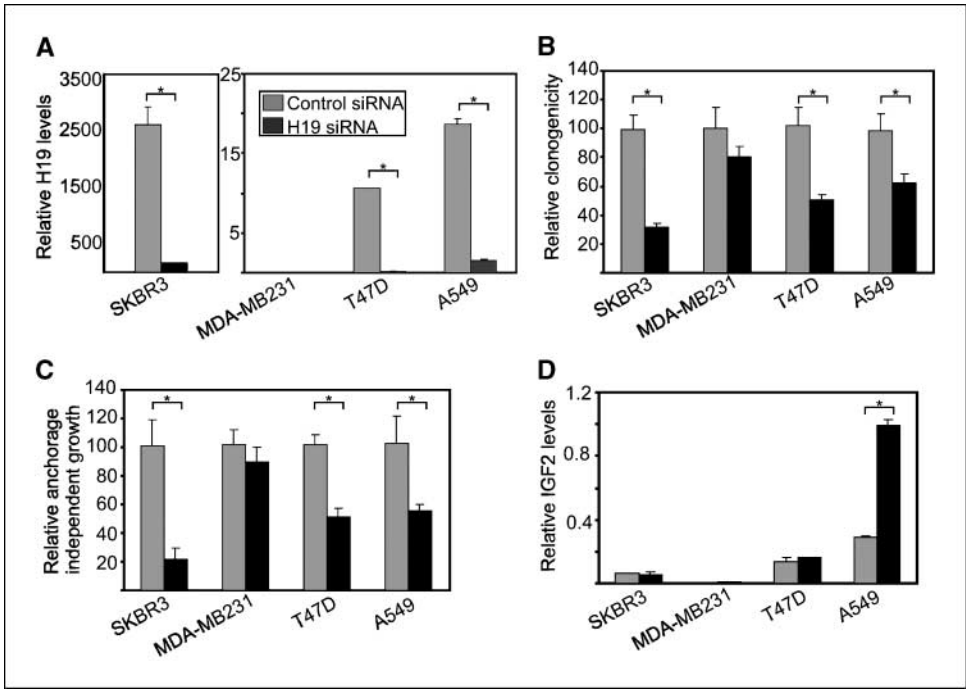

Figure 4. H19 siRNA inhibits cellular transformation. *A,* quantitative RT-PCR assessment of the basal H19 expression and efficacy of H19 siRNA. 36B4 mRNA was used for normalization. The graph of SKBR3 is illustrated separately due to the very high H19 expression. Quantitative RT-PCR was conducted twice in triplicate. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistical significance as assessed using a paired *t* test. *B,* clonogenicity is decreased in response to the ectopic expression of H19 siRNA in cell lines with basal H19 expression. The experiment was conducted two independent times with consistent results; a representative experiment is shown. Experimental results were normalized between the cell lines. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistical significance as assessed using a paired *t* test. *C,* anchorage-independent growth is decreased in response to H19 siRNA. Data were analyzed, and significance test was done as in (*B*). *D,* quantitative RT-PCR analysis of IGF2 expression was conducted as in *A*.

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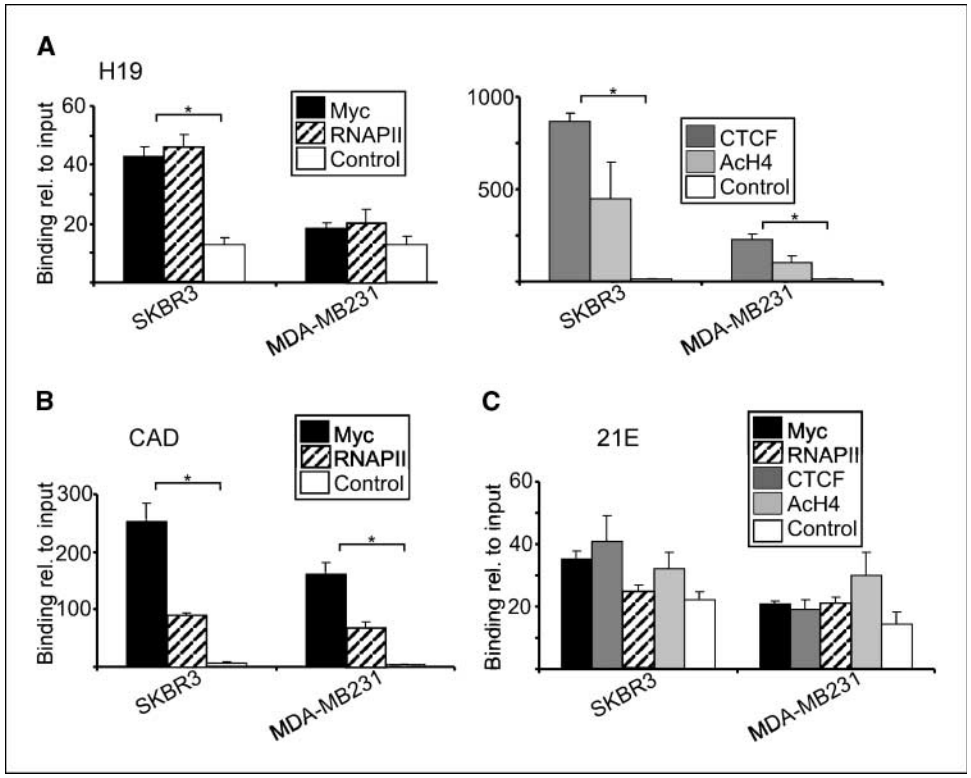

Figure 5. Transcriptional complex binding at the *H19* promoter in SKBR3 and MDA-MB231 cells. *A,* chromatin immunoprecipitation and quantitative PCR analysis of transcriptional components binding the *H19* E-box. *Left,* Myc and RNAPII binding relative to control; *right,* CTCF and AcH4 binding. *B,* chromatin immunoprecipitation and quantitative PCR analysis of Myc and RNAPII binding at the positive control CAD in both cell lines. *C,* chromatin immunoprecipitation and quantitative PCR analysis of negative control E-box binding on chromosome 21. Real-time quantitative PCRs were done and analyzed as in Fig. 2. *, $P < 0.001$, statistical significance as assessed using a *t* test.

tumors, we obtained expression estimates for both genes from a large microarray study of 137 node-negative breast cancer cases. Following data normalization and centering, patients were dichotomized into two equal-sized groups based on H19 expression levels (Fig. 6). Using a two-tailed heteroscedastic *t* test, we show that c-Myc expression was significantly higher ($P = 0.009$) in breast tumors with high H19 levels than those showing lower H19 expression. To show that this is a feature of other tumor types, we characterized the relationship between H19 and c-Myc expression in lung cancers using quantitative RT-PCR in a panel of 240 NSCLCs. Following normalization to a battery of four housekeeping genes, we again dichotomized patients into two equal-sized groups based on H19 expression levels (Fig. 6). Using a two-tailed heteroscedastic *t* test, we again found that c-Myc expression was significantly higher ($P = 0.002$) in tumors with elevated H19 levels than those showing lower H19 expression. To ensure that our results are not artifacts of the statistical analysis, we verified the results using a two-log threshold with the hypergeometric test and again found statistical significance ($P = 0.01$). Both boxplot and spikeplot representations of the data are available as Supplementary Figs. S3 and S4. These analyses show positive correlation of high H19 levels with elevated c-Myc mRNA levels. Selected clinical information is available in Supplementary Tables S4 and S5.

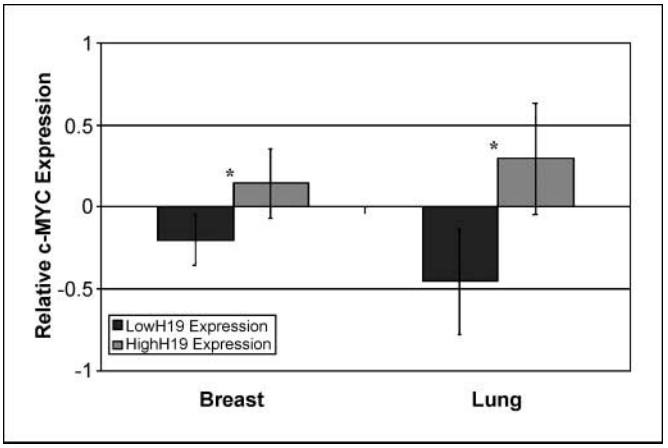

Figure 6. The expression of H19 and c-Myc are related in both breast ($n = 137$) and lung ($n = 240$) tumor samples. For each tumor type, tumors were dichotomized into two equal-sized groups based on H19 expression, and c-Myc expression was compared between the high and low H19 cohorts. *, $P < 0.01$, significance by a two-tailed *t* test. *Columns,* mean; *bars,* 95% confidence intervals.

Discussion

Based on allelic expression and genomic binding studies, we show that c-Myc directly binds to the *H19* promoter and highly up-regulates the transcription of the maternal *H19* allele by recruiting HAT activity. We further show that H19 knockdown, in a panel of breast and lung cancer cell lines, results in the reduction of their tumorigenic phenotype as shown by foci formation and anchorage-independent growth assays. Indeed, a strong association between c-Myc and H19 transcript levels was evident in both primary breast and lung cancer patient material. Taken together, these results indicate that Myc up-regulation of H19 strongly contributes to the tumorigenic phenotype of breast and lung cancer cells.

Our study shows that c-Myc binds to the conserved E-boxes at the *H19* promoter close to the ICR and up-regulates the expression of this ncRNA in MCF10A breast epithelial and established rat fibroblast cell lines. The physical boundary between the ICR and the promoter of *H19* remains undefined; thus, we investigated the mechanism of regulation and show that Myc does not affect ICR

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function or imprinting of this locus, as H19 remains expressed from one allele only. In a parallel study, Lee et al. also found that c-Myc up-regulates H19 in mouse liver *in vivo*.[8] However, their data indicate the strongest binding of Myc at an enhancer downstream of the *H19* promoter in hepatocytes. We did not see significant binding of Myc to this region in MCF10A cells expressing ectopic Myc (data not shown). The distinct binding sites and possibly induction mechanisms used by Myc in human and rat transformed cells compared with murine nontransformed hepatocytes is intriguing and likely is a consequence of cell type differences. Importantly, only the maternal allele of *H19* was regulated by Myc in both studies.

Our results show that c-Myc binds and regulates the maternal *H19* allele strongly supporting the observation that the c-Myc/Max complex is unable to bind methylated E-boxes *in vitro* (36). While this article was in preparation, N-Myc was also reported to selectively bind to unmethylated E-boxes (37). This suggests that E-box methylation is a determinant of c-Myc and N-Myc as regulators of gene transcription. This is further supported by our results showing that the silenced paternal allele did not display binding or regulation by Myc in MCF10A cells, whereas removal of DNA methylation leads to induction of the paternal allele by c-Myc. These data suggest that changes in the DNA methylation patterns, which are common in cancer (38), could alter transcriptional profiles and thus modulate the biological function of Myc.

Our results indicating that c-Myc binds and activates *H19* in an allele-specific manner suggests that additional transcription factors, as well as Myc, may regulate transcription with allelic preference. Recent analyses have shown that allelic variation in gene expression is common even in the nonimprinted genes in mammalian genomes and may affect 20% to 50% of human genes (39). We also show that the main function of Myc at the *H19* promoter is to recruit HAT activity and RNAPII for transcriptional initiation. In cells lacking Myc, basal H19 expression was induced only in response to HDAC inhibition but not to the inhibition of DNA methylation. Similarly, the silencing of $p16^{INK4A}$ can also occur in the absence of DNA methylation through histone modifications (40). Therefore, Myc binds the ICR of *H19* in an allele-specific manner to induce transcription through the recruitment of HAT activity.

Furthermore c-Myc down-regulates IGF2 transcripts in cell lines with detectable IGF2 mRNA. Mechanistically, our data indicate that Myc binding is restricted to the maternal allele ICR and is unlikely to have an effect on IGF2 expression from the paternal allele. Although it has been reported that H19 can repress IGF2 expression (41), we did not consistently observe this cross-regulation in the cell systems studied, suggesting this potential regulatory mechanism may be a cell-dependent and/or tumor type–dependent phenomenon. Surprisingly, our data indicate that Myc binds the E-box in the first intron of *IGF2* to repress transcription. Most Myc repressed genes analyzed to date are regulated through initiator or proximal promoter regions (6–8). It remains to be determined whether E-box-dependent Myc repression is evident and unique to IGF2 or functional at multiple loci. Moreover, it remains unclear whether this potentially novel mechanism is best captured through genomic analysis *in vivo* and not detectable by conventional transient indicator gene analysis methods. Biologically, gene expression analysis of a series of neuroblastoma cell lines indicated that high N-Myc levels correlate with low IGF2 levels (42). However, colon carcinomas display high levels of Myc and loss of *IGF2* imprinting (expressed from both alleles) that is sometimes accompanied by elevated levels of IGF2 (43). This implies that the repression of IGF2 is cell specific or is lost in colon cancer cells. At this time, the significance of IGF2 down-regulation by Myc remains unclear. As a mitogen in many cell types, IGF2 is part of a signaling cascade that is able to induce Myc and Myc repression of IGF2 could function as part of negative feedback loop to control mitogen stimulation. A similar function has been proposed for Myc repression of the platelet-derived growth factor-β receptor (7).

The physiologic role of H19 ncRNA seems to be restricted to the time of embryonic expression (44), and its pathologic role has only recently been investigated. The introduction of H19 into some cell lines resulted in anchorage-independent growth suppression, thus earning H19 tumor suppressor designation (18, 19). Recently, however, H19 expression has been shown to be reactivated in a variety of tumors (20–25). Ectopic expression of H19 in MDA-MB231 and T24P cells increased their growth and tumorigenicity (45, 46), whereas the subsequent knockdown of H19 in MDA-MB231 reduced cell proliferation (35). We show that the knockdown of H19 in SKBR3, T47D, and A549 cells leads to the reduction of their clonogenic ability and decreased anchorage-independent growth. The analysis of H19 and c-Myc expression in primary breast and lung tumor samples indicated that high H19 levels are strongly associated with high Myc transcript levels. This strong association in primary tissue, in combination with the essential role of H19 in transformation, suggests that Myc-induced H19 expression contributes to both tumor etiology and Myc's function as an oncogene.

The function of H19 remains enigmatic. Although knockout *H19* mice seem grossly normal (47), we and others show that H19 plays a role in the tumorigenic phenotype (35, 45, 46). Based on the physical association of the H19 transcript with polysomes, the mechanism of H19 action is thought to be at the level of translational regulation (48). In addition, thioredoxin, a modulator of signal transduction and potentiator of tumorigenesis, was recently identified as translationally up-regulated by H19 (49). Further studies will undoubtedly elucidate other H19-regulated molecules and the role of H19 in both physiologic and pathologic settings. As more transcriptional regulators of H19 are determined, H19 regulation and function will also be better understood. Although this work was in progress, E2F1 was shown to regulate *H19* (35), which suggests that Myc and E2F1 can exert a positive combinatorial control over H19 transcription. It is notable that genes bound by Myc (12–15) and E2F1 (50) display a considerable overlap.

We have thus identified *H19* as a Myc-up-regulated gene that potentiates the tumorigenic phenotype of breast and lung cancer cells. Complex interactions between DNA methylation and histone modifications regulate H19 expression, where the role of Myc is to recruit HAT activity to unmethylated E-boxes and initiate allele-specific *H19* transcription.

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[8] Personal communication.

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TUMORIGENESIS

The silent messenger

A variety of genes are known to be regulated by the transcription factor MYC, but which genes are crucial for its tumorigenic qualities are not clearly defined. Linda Penn and colleagues have found that a non-coding RNA, H19, is a transcriptional target of MYC and the inhibition of H19 transcription in cancer cells suppresses certain cellular characteristics associated with oncogenic transformation.

Despite being first identified as a tumour suppressor, increased expression of H19 is seen in a variety of human tumours, including lung and breast tumours. *H19* resides in the same locus as the insulin-like growth factor 2 gene (*IGF2*), and both of these genes are subject to genomic imprinting — only the maternal allele of *H19* and the paternal allele of *IGF2* are expressed. Through the combined use of RT-PCR, northern blotting and allele-specific chromatin immunoprecipitation techniques the authors were able to show that MYC binds to the regulatory regions of the *H19* and *IGF2* genes. Furthermore, MYC binds to only the promoter of the maternal allele of *H19* to induce transcription, indicating that MYC does not alter the imprinting of this gene.

Short interfering RNAs targeting *H19* in cells derived from lung and breast tumours inhibited anchorage independent growth and colony formation *in vitro* — two well known characteristics of transformed cells. But is there a correlation between the level of MYC expression and H19 expression in these tumours? The authors undertook a large microarray study using 137 node-negative breast cancer samples. Statistical analyses showed that expression levels of MYC were significantly higher in samples with high levels of H19 expression. Similar results were obtained using 240 non-small cell lung cancer samples.

The function of non-coding RNAs is unclear, but it is thought that the H19 transcript might regulate RNA translation. So, MYC might indirectly regulate the expression of a number of proteins in tumour cells through the increased expression of H19.

Nicola McCarthy

References and links

ORIGINAL RESEARCH PAPER Barsyte-Lovejoy, D *et al.* The c-Myc oncogene directly induces the H19 noncoding RNA by allele-specific binding to potentiate tumorigenesis. *Cancer Res.*, **66** 5330-5337 (2006)

Subject: Research Highlight from Nature Reviews Cancer
Date: Fri, 19 May 2006 16:18:44 +0100
From: "McCarthy, Nicola" <N.McCarthy@nature.com>
To: <lpenn@uhnres.utoronto.ca>

Dear Linda,

Hi, I hope you are well. I've written a short highlight article on your Cancer Research paper on Myc, H19 and IGF2, which we plan to publish in the july issue of Nature Reviews Cancer. I've left out much (all) of the discussion and results on Myc and HATs and the methylation side of things and concentrated on the take home messages for the cancer community.

Can you take a look at the attached for me and let me know if I have made any mistakes or if you have any suggested changes.

If you could let me have your comments by 23rd May, that would be great.
With best wishes,
Nicola.

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Appendix 3
DRAFT – to be submitted

Characterizing cMyc MBII and its interaction with TRRAP

Lilia Kaustov,[1] Sigal Katz,[1] Cynthia Ho[1#], Romina Ponzielli[1], Shili Duan,[1] Steve McMahon[3], Michael D. Cole[4], Linda
Z. Penn,[1] and Cheryl H. Arrowsmith[1,2]

[1] *Division of Cancer Genomics and Proteomics, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 610 University Ave, Toronto, Ontario M5G 2M9, Canada*

[2] *Banting and Best Department of Medical Research & Department of Medical Genetics and Microbiology, University of Toronto, 112 College Street, Toronto ON M5G 1L6, Canada*

[3] *The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA*

[4]*Dartmouth College, Molecular and Cellular Biology Program, 7560 Remsen Building, Room 239, Hanover, New Hampshire 03755-3842 USA*

 # Present Address: Samuel Lunenfeld Research Institute, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada

Corresponding authors: CHA – structural biology; LZP – molecular biology.

Abstract

The N-terminal domain (NTD) of cMyc oncoprotein (amino acids 1-262) interacts with several independent co-factors to regulate many critical biological activities, including tumorigenesis. TRRAP is a large 3830 amino acid protein that was isolated biochemically by virtue of its interaction with the highly conserved and functionally critical Myc Box II (MBII) region (amino acids 128-143) of the cMyc NTD. In this study we identify a central region of TRRAP that interacts with the Myc NTD using a novel two-hybrid approach, termed the Repressed Transactivator (RTA) assay. To further delineate the region and investigate the nature of the interaction, we analyzed the Nyc NTD and several TRRAP polypeptides within the Myc-binding domain using nuclear magnetic resonance and circular dichroism. We show that several Myc NTD polypeptides are largely disordered in solution, which is consistent with previous reports of the Myc NTD as well as many other transcriptional activation domains. We also show that a fragment of $Myc_{120\text{-}160}$, which contains the MBII region, forms a complex with a fragment, $TRRAP_{F12}$ that appears to have a folded, globular domain. Ectopic expression of this region of TRRAP, as well as a slightly smaller region $TRRAP_{F8}$, in mammalian 293TV cells shows colocalization with Myc by indirect imunofluorescence and confocal microscopy. Evidence of interaction with endogenous cMyc is shown by co-immunoprecipitation in vivo. Further analysis reveals that this interaction is lost upon mutation of Tryptophan135 (W135) in the MBII region of cMyc both in vitro and in vivo. Thus, we have defined a region of TRRAP which binds to the MBII region of the Myc NTD in a W135 dependent manner.

Introduction

Deregulated expression of the c-myc oncogene is a common feature of numerous human cancers of diverse origin and is often associated with aggressive disease. The underlying mechanisms of deregulation include chromosomal translocation, amplification of the c-myc locus, as well as loss of transcriptional and post-transcriptional regulatory mechanisms controlling the expression and activity of the cMyc protein. [1-3] The product of the human c-myc gene, cMyc, is a 439 residue multifunctional, nuclear phosphoprotein that regulates a wide spectrum of biological activities including cell proliferation, differentiation and programmed cell death. cMyc is thought to orchestrate these many activities by functioning in concert with specific cofactors to regulate gene transcription. The nature of these interactions with Myc, and the regulatory roles of these cofactors in controlling this potent oncoprotein is an area of much interest, as they provide a new and promising avenue for further development of novel anti-Myc therapeutics. [4]

The cMyc protein belongs to a subfamily of transcription factors characterized by two well defined domains at each terminus. The C-terminal domain (CTD) of cMyc consists of a basic DNA binding motif (b) which is adjuacent to two dimerization motifs; the helix loop helix (HLH) and leucine zipper (LZ). Both the HLH and LZ motifs are important for Myc to bind to its obligate bHLHLZ partner, Max. cMyc/Max heterodimers are capable of recognizing and binding DNA targets to activate or repress gene transcription. The mechanism of cMyc/Max interaction with DNA has been well characterized, in part because the structure of the C-terminal bHLHLZ has been solved.[5-9] By contrast, a precise molecular and structural understanding of the cMyc N-terminus has remained elusive, despite the functional importance of this region for all known biological activities of cMyc. Recent efforts have identified a number of important protein interactions at the cMyc N-terminus.[10] The challenge remains to resolve the interactions between the cMyc and its N-terminal partners at a molecular level, to determine how these interactions are regulated, and identify which interactions are essential for transformation. This knowledge can then be exploited for the development of novel anti-cancer therapeutics that target the critical Myc:cofactor interactions to which the tumor has become addicted.

The NTD of cMyc spans amino acids 1 to 262 and contains three regions that are unique to Myc and highly conserved amongst all members of the Myc family of proteins. These

regions are crucial for many biological activities and include Myc boxes I (MBI, amino acids 45 to 63), II (MBII, 128-143) and III (MBIII, 188-199). Residues 1-143, constitute the transactivation domain (TAD), which is sufficient to activate gene transcription when linked to a heterologous DNA-binding domain. Indeed, the TAD is required for cMyc to bind to the basal transcription factor, Tata-binding protein (TBP). The biophysical characterization of the cMyc TAD has been investigated by Circular Dichroism (CD). This analysis showed little or no secondary structural content in the absence of binding partners, which agrees with several studies of other transactivation domains. More recent analysis of a larger fragment of the Myc NTD, residues 1-167, which includes the unstructured cMyc TAD, identified a partially helical region.[11] Evidence suggests the structure and function of the Myc NTD is highly dependent upon interaction with its partner proteins and operates on an induced fit model of interaction.

One of the most intriguing Myc-binding proteins yet described is the large (~430 KDa) ATM/PI3-kinase-related protein termed, transformation-transactivation domain-associated protein (TRRAP). Inhibition of TRRAP expression or function blocks Myc-mediated oncogenesis,[12] establishing an essential role for TRRAP in cMyc activity. Indeed, the activation and oncogenic potency of the individual Myc family members was directly proportional to their ability to bind TRRAP.[13] Moreover the interaction between cMyc and TRRAP has been shown *in vivo* using both exogenous and endogenous protein. Interaction involves the MBII region of cMyc and disruption of a key Tryptophan (W135) residue within MBII was shown to abolish interaction of N-Myc with TRRAP. TRRAP is a component of at least two multiprotein complexes, termed SAGA and TIP60, whose role is to recruit histone acetyl transferase activity with specificity for histone H3 and H4, respectively.[12-14] It was recently shown that the yeast Tra1 and human TRRAP proteins are a target of several transcriptional activators, including E2F, p53, Gal4, E1A, VP16, nuclear receptors as well as acidic activators.[12,15-21] Nipped A has recently been identified as the fly homologue of Tra1/TRRAP and is thought to facilitate assembly of the Notch activator complex.[22] Moreover, TRRAP has been reported to reside at sites of DNA repair. The functional impact of TRRAP binding to these other DNA-binding proteins is not yet fully defined. Taken together, work to date clearly shows that TRRAP holds a critical role in the regulation and

function of cMyc and identifying the region of Myc interaction of this large protein will mark a key advance.

In this study, we investigated the ability of various regions of TRRAP to interact with Myc using a novel two-hybrid assay and identified a large region comprising residues 1665-2030 which is important for Myc interaction. We have further narrowed the region of Myc interaction to a central domain represented by two overlapping fragments ($TRRAP_{F8}$, $TRRAP_{F12}$) that appears to have a folded, globular domain and forms a complex with residues 120-160 of Myc ($Myc_{120-160}$) that includes MBII. This interaction is dependent on W135 in cMyc, a residue that has been shown to be required for both, interaction with TRRAP[23] and Myc-induced transformation activity.[24,25] Moreover, we demonstrated that complex formation between $Myc_{120-160}$ and $TRRAP_{F12}$ is accompanied by a change in protein conformation, consistent with a model in which target factor binding induces a more structured TAD conformation. Importantly, ectopically expressed $TRRAP_{F8}$ colocalizes and co-immunoprecipitates with cMyc in 293TV cells, confirming the interaction of these domains *in vivo*.

Results

Repressed Transactivator Assay defines a Myc interacting region of TRRAP.

To dissect the large TRRAP protein and identify the region that interacts with cMyc, we used an innovative yeast 2-hybrid system for bait proteins that have intrinsic transactivation activity. We have previously used this system, termed the Repressed Transactivator assay or RTA, to successfully investigate Myc NTD interactors.[26,27] In the RTA (Figure 1(a)), the Myc NTD bait is expressed as a fusion protein with the DNA Binding Domain of Gal 4. Gal4 binding sites are located upstream of two reporter genes: ura3 and lacZ in the yeast strain Mav108. Myc activation of ura3 and lacZ results in the death of the yeast when plated on FOA and expression of beta-galactosidase (beta-gal), respectively. Prey proteins are fused to the repression domain of the yeast TUP1 protein. Bait:prey interaction represses reporter gene expression resulting in the growth of yeast on FOA and inhibition of beta-gal activity.

Using the RTA, several large regions of TRRAP were assayed for their ability to interact with the Myc NTD bait.The prey vector containing the positive control TBP, the TRRAP fragments I-III (Figure 1(b)), or control empty vector (pBDH) were cotransformed with the

Gal4-Myc NTD (Figure 1(c)) or the empty pG control vector (data not shown) into yeast and plated onto media with or without FOA. All yeast were able to grow in the absence of FOA (Figure 1(c), upper panel), but in the presence of FOA (Figure 1(c), lower panel), only TRRAP fragment II and TBP were able to confer growth-rescue of yeast expressing the Gal4-Myc NTD. Expression of all fragments in yeast was confirmed by immunoblot analysis (data not shown). Suppression of lacZ expression to levels similar to those of TBP was observed when TRRAP fragment II was co-transfected into yeast with the Myc-NTD (Figure 1(d)). By contrast, cells expressing TRRAP fragments I and III displayed levels of β-gal activity that were comparable to the empty pBDH vector alone. Thus, using the RTA we were able to identify a region of TRRAP (Figure 1, fragment II) that binds the Myc NTD when expressed in yeast in vivo.

We further evaluated the region of Myc that was essential for TRRAP fragment II interaction using the RTA. MBII is necessary for Myc transformation activity and Myc interaction with TRRAP[12]. Interestingly a substitution mutation, W135E within MBII, has been shown to be critical for Myc transformation. Using this specific point mutant we evaluated TRRAP fragment II interaction with the wildtype and W135E MycNTD. Both the wildtype and mutant Myc NTD were able to transactivate the reporter with equivalent activity, leading to the death and growth of yeast in the presence and absence of FOA, respectively (data not shown). Growth rescue in the presence of FOA was achieved with expression of TRRAP fragment II with wildtype MycNTD, but significantly diminished in the presence of Gal4-MycNTD W135E (Figure 1(e)). Growth rescue was achieved as expected when TBP was expressed as prey with both the wildtype and W135E mutant MycNTD baits (data not shown). These data suggest that residue W135 plays a key role in Myc-TRRAP interaction.

Minimal Myc binding region

To further define the Myc-binding domain of TRRAP fragment II and conduct structural characterization of the cMyc/TRRAP complex, a series of smaller TRRAP fragments were cloned. Eleven domain-sized, partially overlapping fragments, spanning and surrounding the Myc binding region of the TRRAP fragment II were tested for expression in *E. coli*, as well as solubility and interaction with cMyc (Figure 2). These fragments were expressed in *E.coli* as polyhistidine fusions and subsequently purified for biophysical analysis. All the fragments

were insoluble when expressed in *E.coli*. To obtain soluble proteins for interaction studies, a refolding protocol (see Materials and Methods) was developed for several TRRAP fragments.

TRRAP fragment II was expressed at very low levels (1mg/1L LB) and was proteolytically degraded into a smaller fragment with a molecular weight corresponding to that of a fragment just larger than $TRRAP_{F12}$ (confirmed by Mass Spectrometry), suggesting the presence of a protease resistant domain. Because of the low expression level of this fragment of TRRAP, we designed and cloned a series of smaller TRRAP fragments (Figure 2). $TRRAP_{F8}$ and $TRRAP_{F12}$ expressed well, but extensive precipitation precluded refolding of these individual polypeptides (5% -10% refolding yield). However, when both $TRRAP_{F8}$ and $TRRAP_{F12}$ were solubilized with denaturant and refolded in the presence of $cMyc_{120-160}$, these proteins remained soluble suggesting that the folding of these two fragments was facilitated by $cMyc_{120-160}$ (40-50% refolding yield).

While this work was in progress Park et al. described the results of their mapping studies to identify the region of TRRAP that interacts with Myc.[28] They evaluated cMyc:TRRAP interaction by ectopic expression of TRRAP fragments in HEK cells followed by Myc co-immunoprecipitation. This analysis revealed that $TRRAP_{1899-2401,}$ but not fragments C-terminal to this region, could weakly interact with cMyc. Using a GST-pull down approach they showed that $TRRAP_{1591-2026}$ could interact with cMyc in vitro. Based on these results they suggested that the minimal region of Myc interaction may reside in $TRRAP_{1899-2026.}$ To directly evaluate whether this region did indeed bind to Myc, we cloned and expressed this fragment in *E.coli* in a similar manner to the other 11 fragments described above. $TRRAP_{F1}$ was the only fragment that could be expressed individually in soluble form. Thus $TRRAP_{F1}$ could also be further evaluated for MycNTD interaction.

Structural analysis of $cMyc_{120-160}$, $cMyc_{1-153}$, $TRRAP_{F12}$, and $TRRAP_{F1}$

To identify the region(s) of cMyc that interact with its regulatory partner, TRRAP, we used circular dichroism (CD) and NMR spectroscopy to analyze the secondary structural characteristics of two cMycNTD and two TRRAP polypeptides. CD analysis of the fragments $cMyc_{120-160}$ and $cMyc_{1-153}$ showed two different secondary structural properties. The CD spectrum of $cMyc_{1-153}$ shows little negative elliplicity at approximately 222 nm and a minimum shifted towards 200 nm which indicates a largely disordered structure with low

helical content (Figure 3(a)). A comparison of the experimental CD spectrum of Myc_{1-153} with that previously published for Myc_{1-143} indicates similar secondary structural properties. Recent analysis of Myc_{1-167} by Fladvad *et al*. suggest that residues 140-157 contribute to the folding of the entire cMyc TAD region. This may explain why our Myc_{1-153} (lacking residues 154-167) had a CD spectrum reflecting significantly less secondary structure than that for Myc_{1-167}.[11] We show $Myc_{120-160}$ which encompasses MBII is partly helical with negative minima near 208 nm and 222 nm. Our CD spectrum of $Myc_{120-160}$ is similar to that of Myc_{1-167} and Myc_{92-167}.[11] This region contains MBII residues 128-143 which are conserved in all Myc family proteins and known to be essential for the Myc/TRRAP interaction.

^{1}H-^{15}N HSQC spectra of $Myc_{120-160}$ (Figure 3(b)) and Myc_{1-153} (data not shown) reflect disordered proteins with very little chemical shift dispersion. As shown in Figure 3(b), $Myc_{120-160}$ fragment has a spectrum that reflects soluble, non-aggregated, partially folded protein with the approximate number of expected cross peaks. Therefore, $Myc_{120-160}$, which contains a conserved structural domain and has favorable solubility properties, was advanced for further Myc/TRRAP interaction studies.

The favorable solubility properties of the $TRRAP_{F1}$ fragment, which corresponds to the previously proposed Myc-binding region, allowed ^{1}H-^{15}N HSQC NMR to be used to study the potential interaction of this polypeptide with cMyc. $TRRAP_{F1}$ was incubated with Myc_{1-153} and analyzed by gel filtration. No aggregation as well as no-coelution were detected for the protein mixture (data not shown). Next, ^{1}H-^{15}N HSQC NMR spectra were acquired for Ul-^{15}N- $TRRAP_{F1}$. The $TRRAP_{F1}$ ^{1}H-^{15}N HSQC spectrum reflects a soluble, non-aggregated, but disordered protein with very little chemical shift dispersion, and narrow peak width (both features of disordered proteins, Figure 3(c)). Addition of Myc_{1-153} had no effect on the spectral features of Ul-^{15}N- $TRRAP_{F1}$ (data not shown). Therefore, we conclude that there is very little or no interaction between $TRRAP_{F1}$ and Myc_{1-153} under these conditions, as a complex could not be identified by either NMR, or by GST pull down assays (see below) at millimolar protein concentrations.

Using CD spectroscopy we have identified a region of TRRAP that appears to have a folded, globular domain ($TRRAP_{F12}$) and is likely to form a complex with the conserved MBII. While the spectrum of $Myc_{120-160}$ alone was characteristic of a mix of helical and random coil conformation, the CD spectrum of $TRRAP_{F12}$ fragment alone had a strong

minimum near 217 nm, characteristic of a substantial proportion of β-sheet structure. The spectrum of the mixture is clearly distinct from those of $TRRAP_{F12}$ and $Myc_{120-160}$ alone with an obvious minimum at 222 nm (Figure 3(d)) indicative of the conversion of a random coil to an α-helical conformation.

Attempts to use NMR spectroscopy to study the interaction between $TRRAP_{F12}$ or $TRRAP_{F8}$ fragments with cMyc, were unsuccessful due to the low solubility and aggregation of TRRAP fragments during NMR sample preparation, under a variety of conditions. As well, efforts to prevent aggregation of $TRRAP_{F12}$ and $TRRAP_{F8}$ fragments alone and in the presence of different Myc polypeptides were unsuccessful. As an alternative strategy, we constructed a number of chimeric proteins with different Myc fragments fused to $TRRAP_{F12}$ and *vice versa* (see materials and methods). The chimera comprising residues 120-160 of Myc followed by $TRRAP_{F12}$ residues expressed very poorly in *E.coli*. Surprisingly, the chimera comprising $TRRAP_{F12}$ followed by the same Myc residues expressed well although it was largely in inclusion bodies. Refolding and purification of the $TRRAP_{F12}/Myc_{120-160}$ chimera resulted in a protein that eluted in the void volume of a Superdex 200 column suggesting non-specific aggregation. Nevertheless, the CD spectrum of $TRRAP_{F12}/Myc_{120-160}$ chimera (Figure 3(e)) showed helical secondary structure conformation with a minimum at 222 nm, in agreement with the CD spectrum of $TRRAP_{F12}$ and $Myc_{120-160}$ mixture (Figure 3(d)).

In addition, we focused on a particular residue within MBII (W135) that has been shown to be required for both interaction with TRRAP and Myc-induced transformation.[12,23-25] To investigate the nature of the role of this amino acid in Myc-TRRAP interaction we mutated it to a charged amino acid, W135E. The CD spectrum (data not shown) of the protein mixture showed no interaction between $Myc_{120-160}$ W135E mutant and $TRRAP_{F12}$ (data not shown) consistent with our RTA (Figure 1(e)) and pull down assay data (Figure 4(d))

In vitro binding confirms Myc-TRRAP minimal domains

An *in vitro* GST pull down assay was performed to evaluate the interaction of $TRRAP_{F8}$, $TRRAP_{F12}$ and $TRRAP_{F1}$ with $Myc_{120-160}$ and $Myc_{120-160}$W135E. $cMyc_{120-160}$ and $Myc_{120-160}$W135E GST fusions were expressed in *E.coli* and purified on Glutathione sepharose beads. The beads complexed with GST fusion $Myc_{120-160}$ were then incubated with metal

affinity purified His tagged $TRRAP_{F12}$, $TRRAP_{F8}$ and $TRRAP_{F1}$ constructs to assess binding. The GST fusion $Myc_{120\text{-}160}$ mutant was incubated with His tagged $TRRAP_{F12}$. The results clearly showed an interaction of $Myc_{120\text{-}160}$ with both $TRRAP_{F12}$ and $TRRAP_{F8}$, but not with $TRRAP_{F1}$ (Figure 4). No interaction was observed between $Myc_{120\text{-}160}$W135E mutant and $TRRAP_{F12}$ (Figure 4(d)). Together, these data indicate that $Myc_{120\text{-}160}$ can bind to $TRRAP_{F8}$ and $TRRAP_{F12}$ and a nonconservative mutation within MBII, W135E, was able to disrupt the binding between these proteins.

$TRRAP_{F8}$ interacts with chromatin-bound Myc in vivo.

We further investigated whether the interaction of $TRRAP_{F8}$ and Myc was detectable in mammalian cells *in vivo*. To this end, 293TV cells were transiently transfected with CMV10 expression plasmid carrying a fusion protein containing a FLAG-tagged nuclear localization signal (NLS) linked in-frame with $TRRAP_{F8}$. To investigate the sub-cellular localization of $TRRAP_{F8}$, we conducted immunofluorescence studies as well as biochemical analyses. Cells were fixed and immuno-stained with polyclonal anti-Myc and monoclonal anti-FLAG antibodies to detect cMyc and $TRRAP_{F8}$ respectively. As shown in Figure 5(a), $TRRAP_{F8}$ and cMyc primarily co-localize in the nucleus of 293TV cells, in a diffuse dot pattern (white arrows in Figure 5(a), panel c). In addition, a less prevalent staining of $TRRAP_{F8}$ is shown in the cytoplasmic compartment, most likely due to over-expression of the protein. For biochemical analysis, cell lysates were harvested and sub-cellular fractions prepared. Equivalent volumes of cytoplasmic, nuclear and chromatin-enriched fractions were separated on a 12% SDS-PAGE gel (Figure 5(b)). In addition to anti-FLAG, immunoblots were probed with anti-tubulin to monitor efficiency of cellular fractionation, and anti-acetylated histone H3 to confirm chromatin enrichment. Interestingly, Flag-NLS- $TRRAP_{F8}$ was highly enriched in the chromatin fractions, yet also evident in the cytoplasmic fraction, in accordance with the immunofluorescence results. To determine if $TRRAP_{F8}$ interacts with full length cMyc *in vivo* after transient co-expression in 293TV cells we conduced co-immunoprecipitation experiments. Cell lysates were immunoprecipitated with anti-Myc antibodies (N-262) followed by immunoblotting with anti-FLAG antibodies to identify FLAG-$TRRAP_{F8}$ protein (Figure 5(c)). As shown in Figure 5(c) lane 4, FLAG-$TRRAP_{F8}$ was specifically co-immunoprecipitated by Myc and not by rabbit IgG (lane 3). Taken together, these observations provide strong evidence of a physical interaction between cMyc

and $TRRAP_{F8}$. This suggests that the $TRRAP_{F8}$ fragment binds to chromatin in conjunction with Myc.

Discussion

A significant advance in understanding the mechanisms of Myc-induced transformation will be achieved by identifying the proteins that interact with the Myc to regulate oncogenic gene transcription. The N-terminus is a major regulatory region responsible for the assembly of the transcriptional machinery[10] which is controlled by binding to a range proteins including coactivators, cytoplasmic proteins, transcriptional regulators and tumor suppressors.[10,29-31] However, little is known about the structure of the N-terminal domain or the biophysical mechanisms related to its ability to transactivate.

Interestingly, recent results using CD indicated that the N-terminal domain shows little to no inherent secondary structure, suggesting that protein-protein interactions are essential for proper folding and function of Myc. Moreover, MBII encodes the most hydrophobic region of cMyc, so it is likely to be either a core organizer for the NTD fold or an interaction motif. In addition, conserved hydrophobic cMyc residues in MBII (W135, F138) have been shown to be required both for interaction with regulatory proteins and for transformation.[23-25] It has been shown that Myc contributes to chromatin remodeling through an MBII dependent interaction with the regulatory protein TRRAP.[32] Inhibition of TRRAP synthesis or function blocks Myc-mediated oncogenesis, establishing an essential role for TRRAP in Myc activity.

In this study, we investigated the ability of various regions of TRRAP to interact with Myc using the novel RTA technology and identified a large region comprising fragment II as important for the Myc interaction. Using CD spectroscopy a folded globular domain of $TRRAP_{F12}$ has been identified and shown to bind $Myc_{120\text{-}160}$ containing the conserved MBII. A more direct assay of protein-protein interactions using the GST pulldown technique in combination with gel filtration was used to map the Myc binding domain on TRRAP. The minimal region, $TRRAP_{F8}$, exhibited binding to $Myc_{120\text{-}160}$. The increased structure and higher stability in the $Myc_{120\text{-}160}$ and $TRRAP_{F12}$ complex compared to that of the individual polypeptides, suggest a folding-on-binding effect and interaction between these proteins, which is consistent with an induced-fit model of protein-protein interaction.

Applying site directed mutagenesis we have shown that a particular residue within MBII (W135) is essential for the Myc-TRRAP interaction. This could be due to either a destabilizing affect on the structural integrity of TRRAP-bound MBII, or direct participation of W135 in the Myc-TRRAP interaction. Thus, consistent with previous reports, we conclude that MBII sequence is required for efficient interaction of the Myc NTD and TRRAP. In addition, our immunofluorescence and immunoprecipitation results provide strong evidence of an interaction between cMyc and TRRAP in the nuclear, chromatin-enriched compartment of mammalian cells. Indeed, ectopically expressed TRRAP colocalizes with cMyc in 293 TV cells. As well $TRRAP_{F8}$ resides in the chromatin-enriched fraction, along with Myc, and is co-immunoprecipitated with endogenous Myc from mammalian cells. Thus, we provide evidence of a physical interaction between cMyc and $TRRAP_{F8}$ from analyses conducted both in vitro (CD, GST-pull down) and in vivo (RTA in yeast, co-IF and co-IP in mammalian cells).

Our work extends a previous report using GST pull down assays to show that Myc and $TRRAP_{1591-2026}$ can interact. Interestingly, our results are inconsistent with previous co-immunoprecipitation data showing Myc and $TRRAP_{1899-2401}$ can interact. It may be that another Myc-binding region resides in this latter portion of TRRAP, however our results are not supportive of this possibility. We show that Myc does not interact with $TRRAP_{F1}$ in vitro, and a region of TRRAP corresponding to fragment III (Fig 1) did not interact with the Myc NTD in the yeast RTA assay in vivo. Our preferred model is that the weak interaction observed between Myc and $TRRAP_{1899-2401}$ in mammalian cells may be a consequence of indirect binding of Myc to another transcriptional activator that binds TRRAP in this region. Indeed, the large TRRAP protein interacts with several transcriptional activators through distinct regions of interaction and has been suggested to bridge functional binding interactions between different activators.[33] It will be interesting to further dissect which activator(s) binds to $TRRAP_{1899-2401,}$ as previous results have shown ectopic expression of this region can prolong the doubling time of IRM-5 neuroblastoma cells three-fold. For example, p53 has been shown to bind $TRRAP_{1992-2370}$ and may be one of many candidate activators that bind $TRRAP_{1899-2401.}$

Taken together, the binding and structural studies presented in this manuscript indicate that $Myc_{120-160}$ can interact specifically with $TRRAP_{F8}$ and that this interaction is

accompanied by changes in protein conformation that are consistent with the induction or stabilization of secondary structure conformation in the cMyc polypeptide. The present results thus suggest that $Myc_{120\text{-}160}$ contributes significantly both to folding and binding properties, and may be required for full biophysical and biological functionality of the cMyc TAD region. The specific interaction of $Myc_{120\text{-}160}$ and $TRRAP_{F8}$ provides a novel protein-protein interaction that is an attractive target for therapeutic intervention in cMyc activated cancers. These results establish the basis for subsequent biochemical and structural analyses of the Myc/TRRAP complex leading to a more comprehensive view of the cMyc regulatory network and will provide new insights into understanding how cMyc function is regulated by interaction with the transcriptional coactivator TRRAP.

Materials and Methods

Cloning, expression and purification

The coding regions for TRRAP fragments and two cMyc fragments, 120-160 and 1-153, were PCR amplified from human TRRAP cDNA and human c-myc cDNA respectively, and subcloned into the pET15b expression vector (Novagen) at 5'-NdeI site and 3'-BamHI site. TRRAP-Myc chimera, $TRRAP_{F12}$ /$Myc_{120\text{-}160}$ and $Myc_{120\text{-}160}$/ $TRRAP_{F12}$, were prepared using the same vector. All fragments were expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). For large scale production, cells were grown at 37 ^{o}C until $A_{260\ nm}$ of approximately 1.0 was achieved and protein expression then induced with 1 mM IPTG for four hours prior to harvest. These highly expressed proteins were purified using metal affinity chromatography under native conditions for the $TRRAP_{F1}$ and in the presence of guanidinium chloride (GnCl) for cMyc and other TRRAP fragments. The latter underwent subsequent refolding by rapid dilution and dialysis against buffer containing 3M GnCl followed by overnight dialysis against the same buffer without GnCl. All protein concentrations were determined by Bradford assay. All buffers contained 2mM Benzamidine, 0.5mM PMSF and 0.5mM TCEP.

GST pull down assay

For TRRAP binding assays, purified $TRRAP_{F1}$, $TRRAP_{F8}$ and $TRRAP_{F12}$ HIS-tagged proteins were incubated with GST-fusion $Myc_{120\text{-}160}$ and $Myc_{120\text{-}160}$W135E bound to the GST beads at 4^{o}C for 1hr. After extensive washing with assay buffer, bound proteins were eluted with 20mM reduced glutathione and detected by SDS-PAGE and Coomassie staining.

CD measurements

CD scans were performed on AVIV 62DS CD spectrometer. Protein concentrations used for CD were 0.1-0.15mg/ml. An equimolar solution of $Myc_{120\text{-}160}$ and $TRRAP_{F12}$ was used for the mixed spectra.

NMR spectroscopy

All NMR spectra were recorded at 25^{o}C on Varian INOVA 600 MHz or 500 MHz spectrometers equipped with room temperature pulsed field gradient triple-resonance probe. The final NMR samples contained 90%H_2O/10%D_2O with a protein concentration ranged between 0.2-0.4 mM. Two-dimensional, gradient-enhanced HSQC spectra were acquired on uniformly[29] N labeled proteins. The spectra were processed with NMR Pipe software package.

Immunoprecipitation and Immunoblot

293TV were transfected using standard calcium phosphate method, Hela cells were transfected using Fugene reagent (Roche). For co-immunoprecipitation 1mg of whole cell lysates were prepared using lysis buffer A (15mM Tris-HCL pH 7.5, 500mM Nacl, 0.35% NP-40, 5mM EGTA, 5mM EDTA) and incubated with 10μl of anti-Myc (rabbit sera) or IgG (Santa Cruz) for 3 hours followed by incubation with 30μl of G-sepharose beads (Santa Cruz) for an additional hour. After 5 washes in Buffer B (25mM Tris-HCL pH 7.5, 250mM Nacl, 0.5% NP-40, 1mM EDTA, 10% glycerol, 1mM DTT) immunoprecipitates were analyzed by Immuno-blotting The proteins were resolved by 12% SDS-PAGE and subjected to immunoblot analysis using anti-cMyc (9E10) and anti-FLAG monoclonal antibodies (M2; Sigma).

Chromatin association assay

293TV cell transiently expressing CMV10-FLAG- $TRRAP_{F8}$ were processed as described by Wysocka et al.[34] Briefly, trypsinized cells were washed with PBS, resuspended in 10mM HEPES pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.34M sucrose, 10% glycerol, 1mM DTT. Cells were lysed with 0.1% Triton X-100 and centrifuged to obtain cytosolic supernatant fractions and nuclear pellets. Nuclear pellets were lysed with 3mM EDTA, 0.2 mM EGTA, 1mM DTT. Resulting pellets and supernates, corresponding to

chromatin and nuclear fractions respectively, were analyzed with cytosolic fractions by Immuno-blotting.

Immunofluorescence

Transiently transfected 293TV cells were fixed in 3% paraformaldehyde, permeabilized in 0.1% Tween 20/PBS, and incubated with anti-cMyc (1:500, N-262) or anti-FLAG (1:50, M2) antibodies. Cells were washed, incubated with Alexa-488 anti-mouse or Alexa-568 anti-rabbit (Molecular Probes, Inc.) secondary antibodies and processed for confocal microscopy.

Acknowledgements

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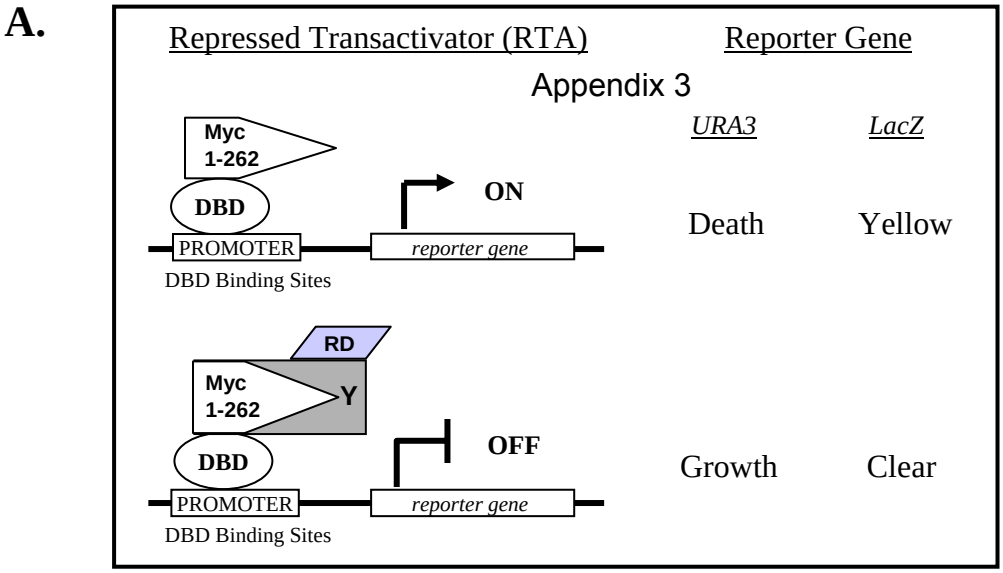
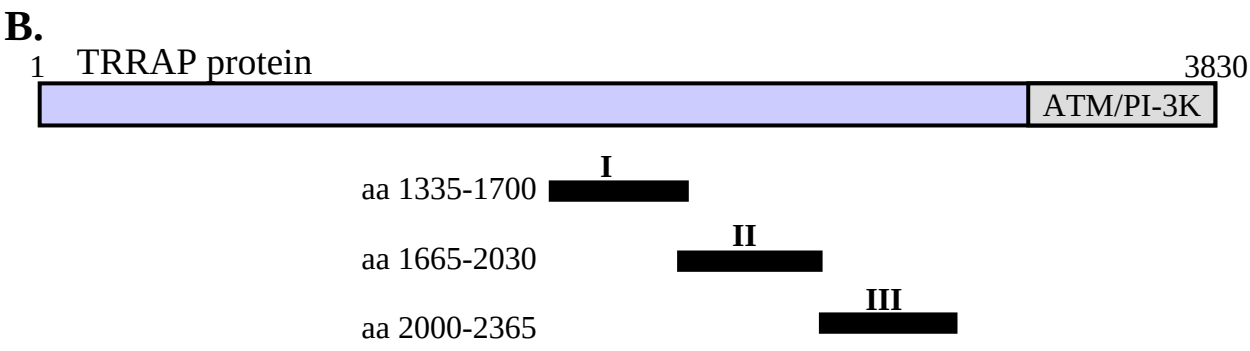
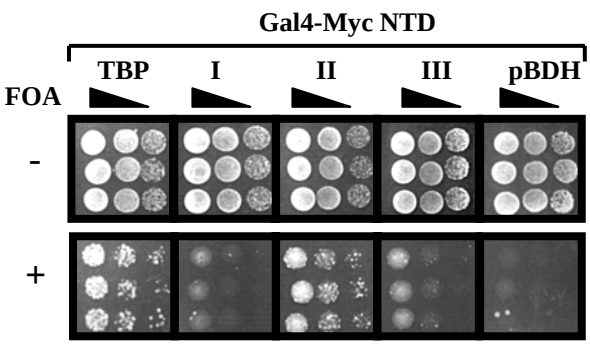
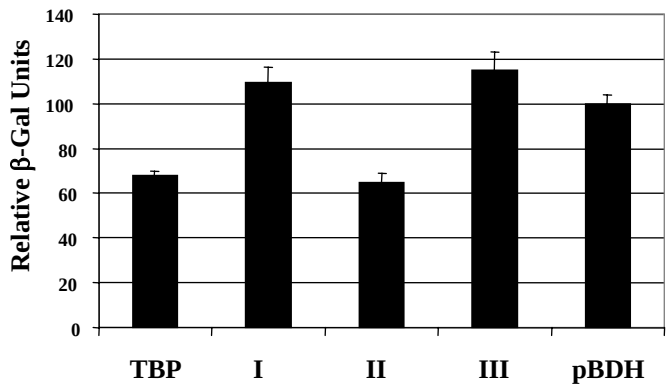
Fig. 1









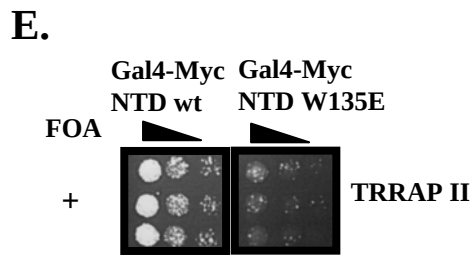


Figure 1: TRRAP fragment II (aa 1665-2030) interacts with Gal4-Myc NTD. (A) The RTA system was used to better define the region of TRRAP that specifically interacts with c-Myc N-terminal domain (Myc 1-262). Growth on FOA and inhibition of LacZ expression are indications of interaction between the bait and prey.**(B)** Schematic representation of TRRAP fragment I (aa 1335-1700), fragment II (aa 1665-2030) and fragment III (aa 2000-2365) used in the RTA. Position of partial TRRAP fragments is outlined using black lines.**(C)** Gal4-Myc NTD bait was co-transformed into yeast cells with TBP or TRRAP fragments I, II, III or a vector containing repression domain alone (pBDH). Resulting colonies were plated onto +/-FOA media for comparison of growth rescue. Only Yeast cells co-transformed with Fragment II and TBP together with MycNTD grew on FOA containing media. **(D)** Comparison of TBP and Fragment II suppression of *lacZ* expression by liquid β-galactosidase assay**.(E)** Point mutation at W135 (W135E), severely affected the interaction of Gal4-Myc NTD with the TRRAP fragment II.

Fig. 2

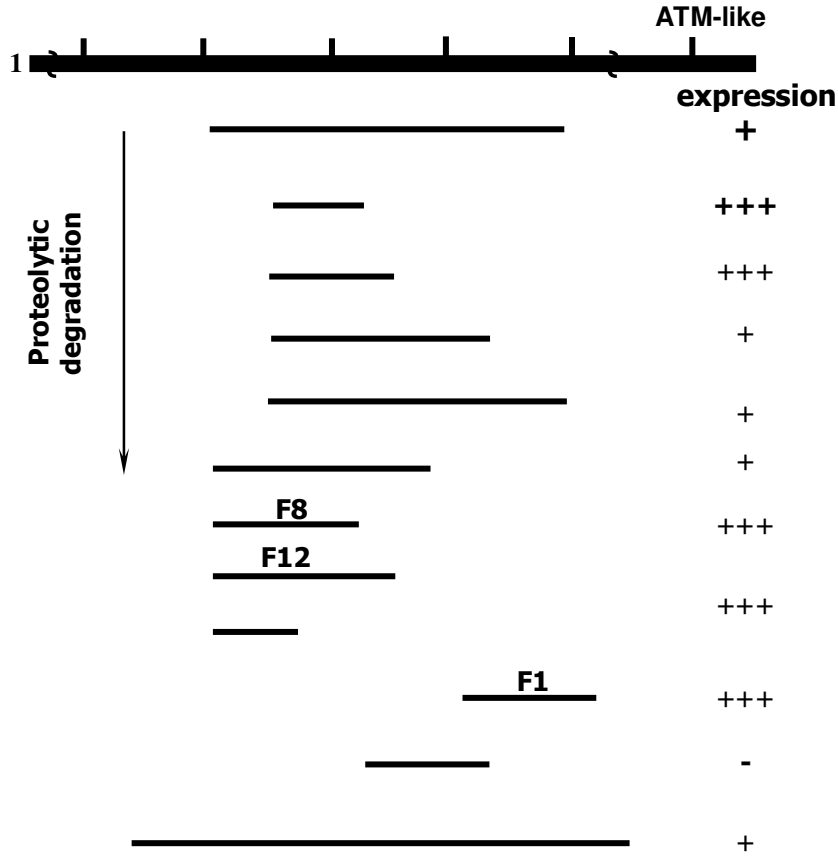


Figure 2. TRRAP central region expression constructs. These constructs were expressed as polyhistidine fusions in E.Coli BL21 with the indicated expression levels. (+) low levels of expression (1mg/1L LB), (+++) good levels of expression (8-12mg/1L LB), (-) no expression.

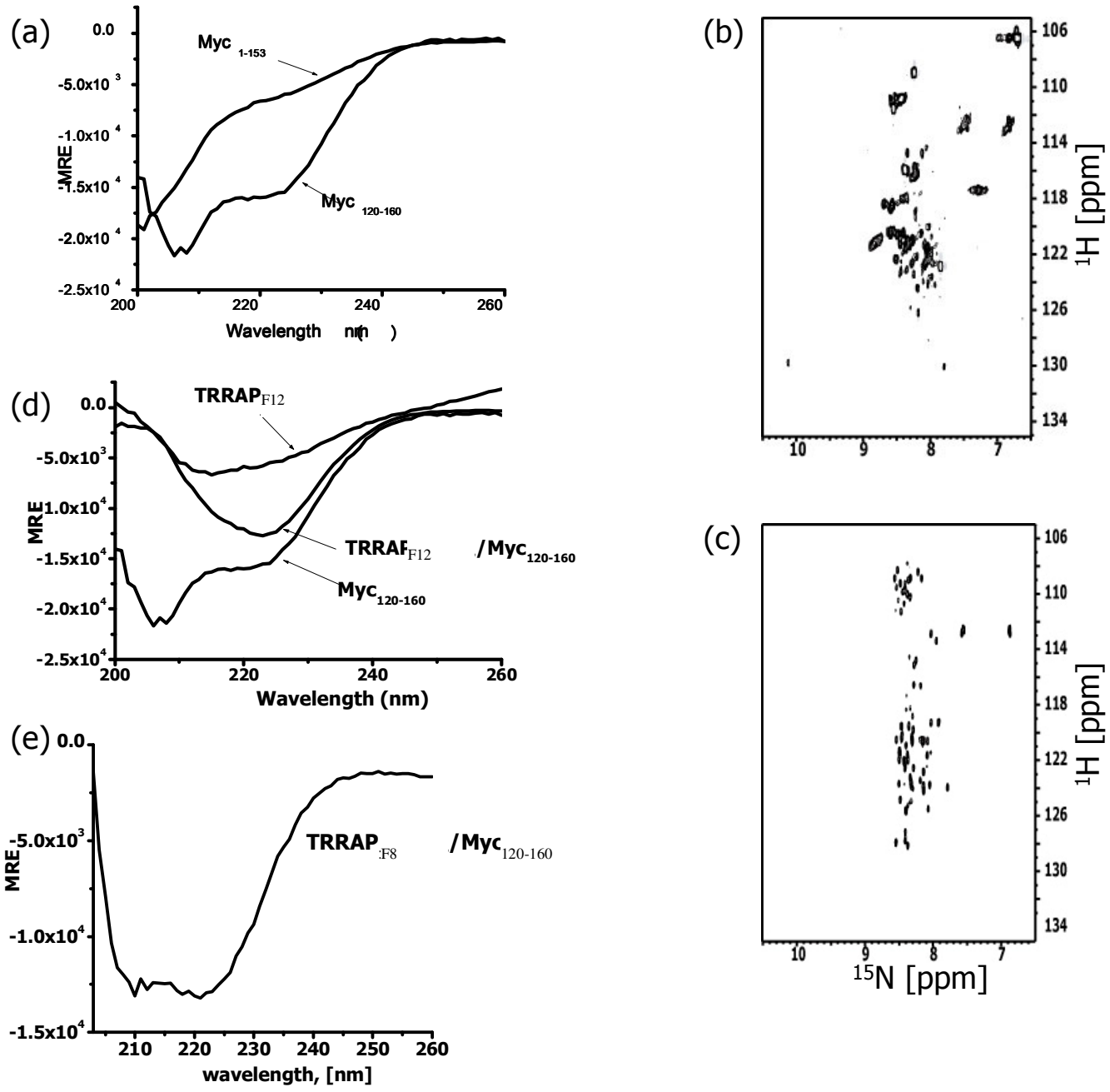


Fig. 3

Figure 3. Secondary structure characterization of c-Myc TAD and TRRAP proteins: (a) CD spectra of c-Myc_{1-153} and c-$Myc_{120-160}$. [15N-1H]HSQC spectrum of: (b) c-$Myc_{120-160}$ and (c)$TRRAP_{F12}$. (d) Interaction of c-$Myc_{120-160}$ with TRRAP causes a conformational change. The CD spectrum was measured for $TRRAP_{F12}$ and c-$Myc_{120-160}$ alone and for a mixture of two proteins, $TRRAP_{F12}$ and $Myc_{120-160}$. (e)The CD spectrum of $TRRAP_{F12}$/c-$Myc_{120-160}$ chimera shows helical secondary structure conformation with a minimum at 222 nm.

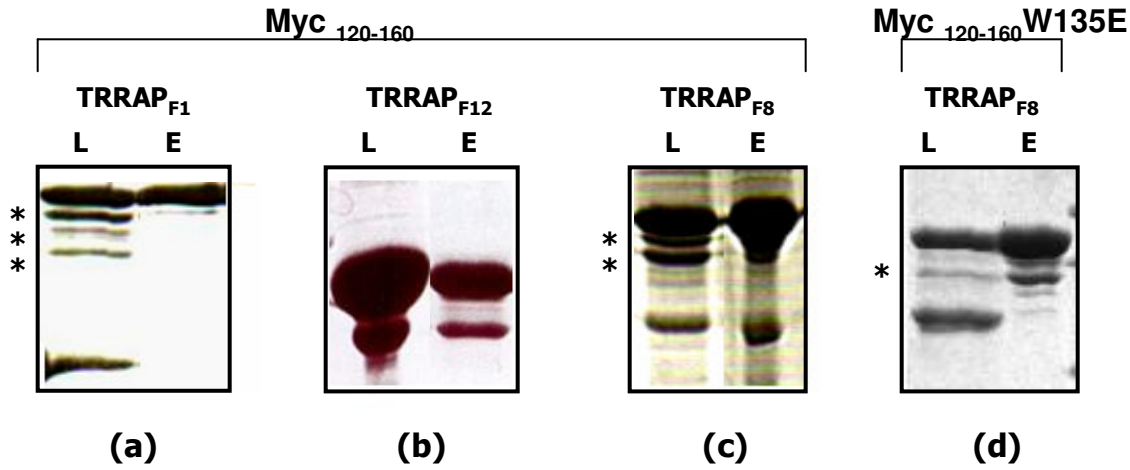


Fig. 4

Figure 4. $TRRAP_{F12}$ and $TRRAP_{F8}$ mediate c-$Myc_{120-160}$ binding. GST-fused c-$Myc_{120-160}$ was bound to glutatione sepharose beads (GST) and co-incubated with His-tagged:(a) $TRRAP_{F1}$; (b) $TRRAP_{F12}$, (c) $TRRAP_{F12}$ constructs (L). (d) $Myc_{120-160}$w135E$TRRAP_{F8}$ mutant was bound to GST beads and co-incubated with $TRRAP_{1690-1877}$ construct (L). After washing with PBSx1 buffer and elution with reduced glutatione buffer (E), interacting proteins were subjected to SDS PAGE analysis. (*) indicates degradation products.

Fig. 5

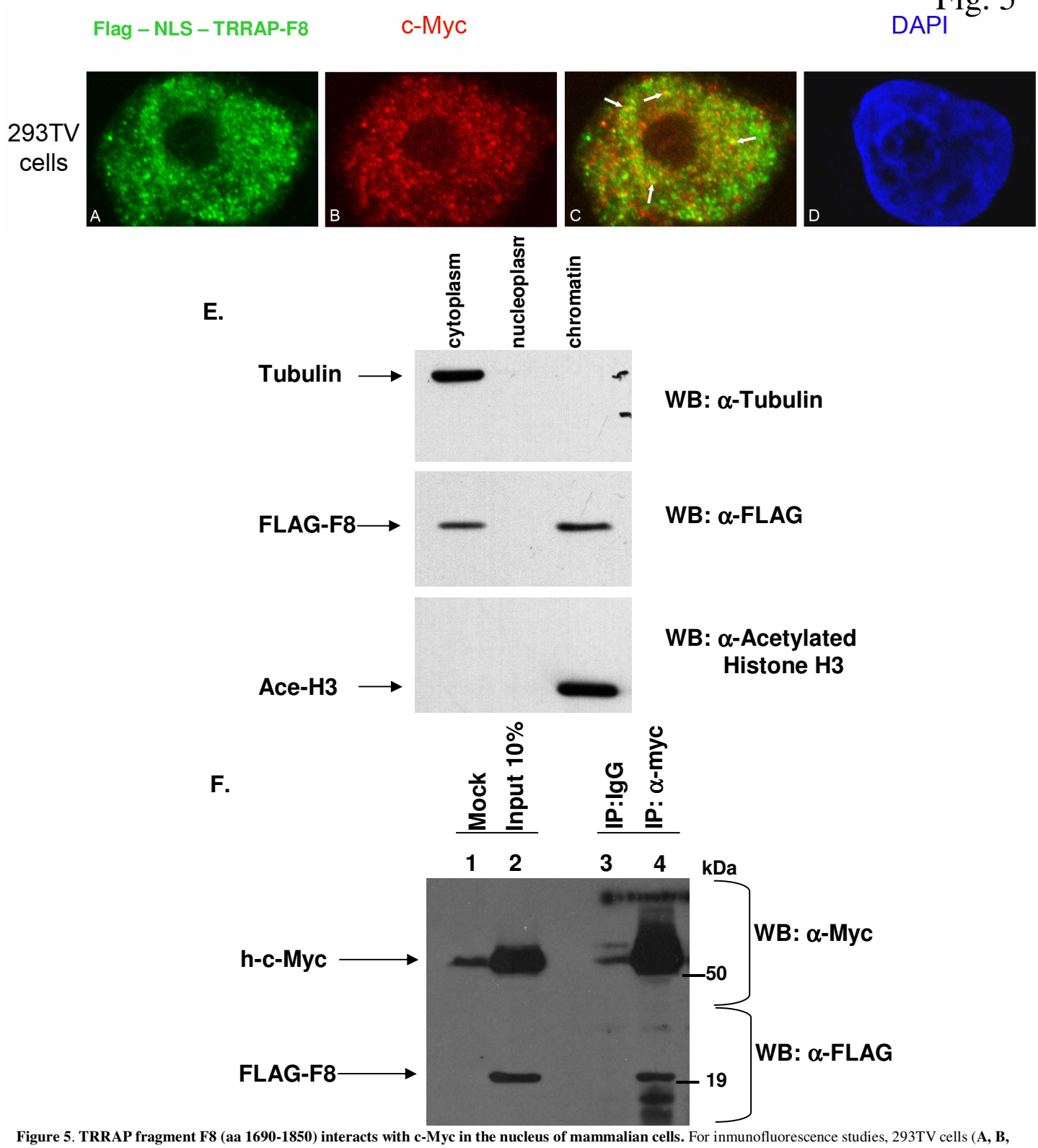


Figure 5. **TRRAP fragment F8 (aa 1690-1850) interacts with c-Myc in the nucleus of mammalian cells.** For inmunofluorescence studies, 293TV cells (**A, B, C, D**) were transiently transfected with CMV10-F8 and pcDNA3-c-Myc for 24hs. Cells were fixed, stained with anti-FLAG monoclonal antibody (M2, Sigma) and rabbit polyclonal anti-Myc antibodies (N-262, Santa Cruz), and processed for confocal imaging. Green immunofluorescence staining shows nuclear localization of F8 and some cytoplasmic localization (**A**). Similar nuclear localization of c-Myc was shown by red fluorescence (**B**). Merged fluorescence images indicate nuclear co-localization of F8 and c-Myc (white arrows in **C**) as well as some cytoplasmic co-localization. Dapi staining shows localization of the nucleus (**D**).
(E) 293TV cells transiently transfected with CMV10-F8 plasmid containing nuclear localization signal (NLS) were harvested and sub-cellular fractions prepared. Equivalent protein amounts of cytoplasmic, nuclear and chromatin subfractions were separated on 12% SDS-PAGE gel. The blot was probed with anti-FLAG then stripped and subsequently re-probed with fractionation control anti-Histone H3 and anti-alpha tubulin antibodies.**(F)** 293TV cells were transiently transfected with CMV10-F8 and pcDNA3-c-Myc. Total cell extract was prepared 48h after transfection and was subjected to immunoprecipitation with anti-c-Myc antibodies (N-262) or rabbit IgG. Co-immunoprecipitated F8 was visualized after SDS-PAGE and immunoblotting with anti-FLAG antibodies (bottom). The upper part of the blot was probed with anti-Myc antibodies (top); Input control (10%) is shown in the second lane while cell extract of mock transfection is shown in the first lane. Migration of FLAG- F8 and c-Myc is indicated.

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Cancer therapeutics: Targeting the dark side of Myc

Romina Ponzielli [1], Sigal Katz [1], Dalia Barsyte-Lovejoy [1], Linda Z. Penn *

Division of Cancer Genomics and Proteomics, Ontario Cancer Institute/Princess Margaret Hospital, Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, Ont., Canada M5G 2M9

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Abstract

The potent Myc oncoprotein plays a pivotal role as a regulator of tumorigenesis in numerous human cancers of diverse origin. Experimental evidence shows that inhibiting Myc significantly halts tumour cell growth and proliferation. This review summarises recent progress in understanding the function of Myc as a transcription factor, with emphasis on key protein interactions and target gene regulation. In addition, major advances in drug development aimed at eliminating Myc are described, including antisense and triple helix forming oligonucleotides, porphyrins and siRNA. Future anti-Myc strategies are also discussed that inhibit Myc at the level of expression and/or function. Targeting the dark side of Myc with novel therapeutic agents promises to have a profound impact in combating cancer.

Keywords: Myc; Max; Therapeutics; Deregulation; Transformation; Target genes; Protein-protein interactors; siRNA; Antisense oligonucleotides; ChIP-chip; Cancer

1. Introduction

These are exciting times for clinicians and scientists working at the front line of cancer research. In recent years, our understanding of the genetic etiology and molecular dependence of cancer has advanced to the point where we can begin to exploit this knowledge for the design of novel, effective, anti-cancer therapeutics. Several new classes of anti-cancer agents have been developed to target pathways that are essential for cancer cell growth and survival. To date, the effort has been primarily focused at targeting growth factor receptors which has led to achievements, such as Gleevec (Imatinib, STI-571), a small molecular weight kinase inhibitor, and Trastuzumab (Herceptin), an antibody reagent that targets the HER-2/neu receptor [1–4]. These agents highlight the merits of molecular targeting and provide an important proof-of-concept that targeted therapeutics can be successfully developed and applied to patient care. However, activating mutations can occur at multiple independent points along oncogenic signalling cascades, so targeting cell surface receptors restricts the range of potentially sensitive tumours. Downstream transcription factors that directly control the transformation program provide an alternative target that may be activated in a broader spectrum of cancers. The Myc transcription factor is one of the most potent and frequently deregulated oncoproteins in human cancers [5,6]. Multiple extracellular and intracellular signalling cascades converge to regulate the Myc oncogene (Fig. 1) making it an especially attractive target in the control of transformation.

In this review, we focus on the most recent advances in understanding the molecular mechanisms of Myc function in the etiology of human cancers. Special emphasis is placed on Myc interactions with other proteins and its gene-regulatory mechanisms. As well, we provide an overview of the past, present and potential future strategies used to target Myc.

* Corresponding author. Tel.: +1 416 946 2276; fax: +1 416 946 2840.
E-mail address: lpenn@uhnres.utoronto.ca (L.Z. Penn).
[1] These authors contributed equally to the work.

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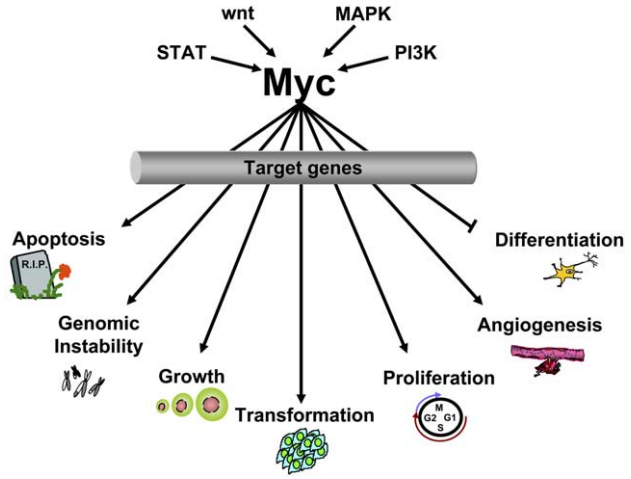

Fig. 1. The regulatory network of Myc. Myc deregulation is a hallmark of many cancers and occurs as a consequence of activation of one or more signalling pathways that induce Myc expression and function as a regulator of gene transcription. These include mitogen activated protein kinases (MAPK) [192–194], phosphatidylinositol-3 kinase (PI3K) [195,196], wnt-TCF/LEF pathway [197], and signal transducer and activator of transcription (STAT) [198] pathways. The target genes regulated by Myc orchestrate the many biological activities attributed to Myc, including apoptosis, genomic instability, growth, transformation, proliferation, angiogenesis and blocking differentiation.

1.1. Myc expression and biological activities

Members of the *myc* family that show oncogenic activity in human cancers include *c-myc*, *MYCN* and *MYCL1*. In a normal physiological state, the protein products of all three genes are expressed during fetal development, whereas only c-Myc protein is expressed in adult tissues. c-Myc protein levels, in non-transformed cells, are induced or suppressed by virtually all signalling cascades bearing proliferative and anti-proliferative cues, respectively. Mitogen stimulation induces c-Myc as an immediate-early response gene, whose expression is essential and sufficient for G1/S progression [7–9]. c-Myc also plays a role in G2/M transition, making it one of the key players in cell cycle regulation [10]. As such, it is important that *c-myc* mRNA and protein have a very short half-life (20–30 min) and are tightly regulated. c-Myc expression is normally rapidly responsive to environmental cues and has been dubbed, "the intracellular sentinel of the extracellular milieu" [11]. By contrast to the highly regulated state of c-Myc and the absence of N-myc and L-myc expression in normal cells, cancer cells often harbour deregulated expression of any one of these three *myc* oncogenes [11].

The founding member of the family, *c-myc*, was first shown to be an oncogene when it was identified as the transduced *v-myc* gene of the transforming avian myelocytomatosis retrovirus [12]. Deregulated c-Myc expression was subsequently shown to be prolific in human cancers. In 100% of Burkitt's lymphoma, c-Myc is translocated with an immunoglobulin enhancer that drives high levels of constitutive *c-myc* mRNA and protein expression, which is instrumental in initiating the disease [13]. Translocations involving the c-Myc locus have also been reported in several additional tumours, including diffuse large cell lymphoma, T-cell acute lymphocytic leukaemia and multiple myeloma [14]. Amplification of c-Myc and/or deregulated expression is evident in many tumours including melanomas and carcinomas of the breast, prostate and colon [5,6,14]. Amplification of *MYCN*, is a hallmark of neuroblastoma [15,16], while *MYCL1* is amplified in ovarian cancer [17]. All three transforming members of the *myc* family can be amplified in non-small cell lung carcinoma [14]. Importantly, in recent years it has become clear that deregulation is not restricted to gross genetic abnormalities of the *myc* gene family, such as translocation or amplification, but can also occur as a consequence of direct or indirect mutations of regulatory molecules controlling *myc* gene expression [11]. Thus, deregulation of Myc expression is evident in numerous human cancers of diverse origin and can result from mutations at one or multiple levels of regulation. Unless otherwise stated, Myc will refer to data described for c-Myc, but these results are often relevant for the highly similar N-Myc and L-Myc oncoproteins.

In response to signals from the cellular environment, Myc can regulate a broad variety of distinct biological activities (Fig. 1). In addition to Myc driving cell proliferation, growth, and transformation, deregulated Myc has also been shown to increase apoptosis, genomic instability, and angiogenesis as well as block differentiation [18–28]. The prevailing model is that Myc controls such a disparate set of activities by regulating distinct cohorts of target genes that then orchestrate each activity. One of the major gaps in the field is linking Myc function, as a transcriptional regulator, to the wide-range of biological activities controlled by Myc [29]. In the following sections we provide an overview of the enormous effort from several labs to identify the target genes regulated by Myc and to uncover the protein–protein interactions that are essential for Myc to function as a transcription factor in transformation.

1.2. Myc structure

Traditionally the Myc protein has been divided into an N-terminal domain (NTD) involved in transactivation and transrepression; and a C-terminal domain (CTD) that is critical for DNA binding. Myc is a transcription factor of the basic helix-loop-helix leucine zipper (b-HLH-LZ) superfamily [30]. The C-terminus harbours both the primary nuclear localisation signal and the basic motif required for binding to the CACGTG E-box DNA recognition sequence (Fig. 2)

[31,32]. The HLH-LZ domain, essential for all known Myc activities, is the heterodimerisation domain that is required for Myc to bind to its primary partner protein, Max (Myc associated protein X).

The N-terminus is a major regulatory region responsible for assembly of the transcriptional machinery [11]. Within the N-terminus there are several highly conserved sequences termed Myc boxes which, together with the C-terminal b-HLH-LZ, define the Myc family of proteins (Fig. 2). Although Myc box I (MBI) is required for gene activation, deletion of this region only partially abolishes the transforming ability of Myc [11,33]. Myc box II (MBII) is essential for the ability of Myc to transform, drive cell proliferation, inhibit differentiation, repress gene transcription, and activate certain target genes [11]. Recently, a third conserved region of Myc has been described, Myc Box III (MBIII), that plays a role in transformation, lymphomagenesis and apoptosis [34]. Interestingly, recent results using circular dichroism indicated that the N-terminal domain shows little to no inherent secondary structure, suggesting that protein–protein interactions are essential for proper folding and function of Myc [35,36]. From a therapeutic perspective this observation offers hope that specific inhibitors can disrupt unique points of interaction between Myc and its binding partners thereby inhibiting transformation. This may offer a novel approach to targeting oncogenic Myc in a tumour-specific manner.

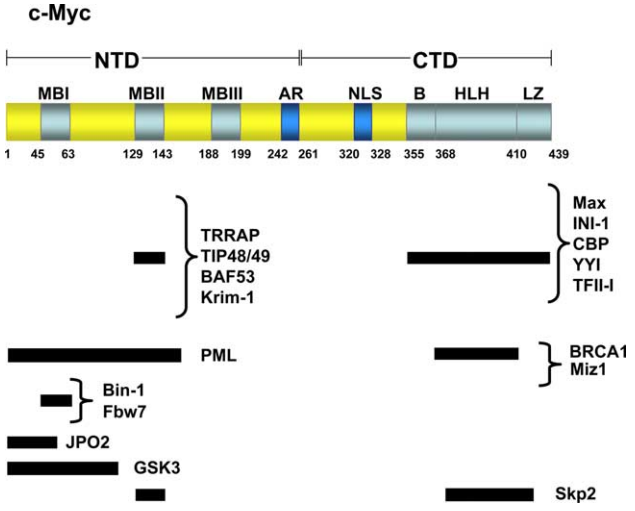

Fig. 2. The structural domains of human c-Myc and their link to protein–protein interactions. c-Myc contains at least six regions which are highly conserved between Myc paralogs and orthologs. The Myc N-terminal domain (NTD) is defined as amino acids 1–262 and contains Myc Box I (MBI), Myc Box II (MBII) and Myc Box III (MBIII) and the acidic region (AR). The Myc C-terminal domain (CTD) is defined as residues 263–439 and contains the primary nuclear localisation signal (NLS) and the basic helix-loop-helix leucine zipper domains (B-HLH-LZ). The regions of c-Myc necessary for the interaction of the specific proteins are shown.

2. Myc interacting proteins

For Myc to hold its extensive role in the control of cellular function, a network of key binding proteins is required (Fig. 2). The identification of this network started with the discovery of Max over a decade ago by screening a human cDNA library with a radiolabeled fusion protein containing the Myc CTD [37]. In recent years, several additional Myc-binding proteins have primarily been identified using biochemical and two-hybrid methodologies. The Myc–Max interaction is already being explored as a therapeutic target, while inhibitors to other key interacting proteins more recently identified, such as TRRAP, have yet to be investigated. A new array of interactors has been identified in recent years, but their role in Myc dependent transformation and the effect of their disruption needs to be further evaluated before they are justifiable targets for therapeutic intervention.

2.1. Myc and Max: from structure to biology

Myc activity is entirely dependent upon dimerisation with Max, an abundant, ubiquitously expressed b-HLH-LZ protein. Recently, the co-crystal structure has been solved and shows that homodimers of Max are held together by polar interactions alone, while Myc–Max heterodimers are stabilised by charged interactions [38]. This results in Myc–Max dimers forming more easily than Max-Max dimers. The crystal structure shows that Myc–Max dimers are stabilised by hydrophobic and polar/charged interactions via helices in the LZ region. Two positively charged residues in Myc form a tetrad with Max, and these two pairs of hydrogen bonds alone control heterodimer specificity with Max. This difference between charged and polar residues also explains the disfavour of Myc–Myc homodimers, caused by electrostatic repulsions between the complementary residues [38,39]. Perhaps one of the most interesting findings from the crystallographic data is the formation of a tetramer with two Myc–Max heterodimers oriented head to tail of the LZ, each binding a DNA E-box. It has been proposed that biologically they may bind widely separated E-box sequences; however, the crystal structure reveals no specific hydrogen bond interactions to stabilise this tetramer [38]. Another function of the heterotetramer might be the formation of a platform for assembly of additional protein factors such as Miz-1, INI1 and BRCA1 which bind to the b-HLH-LZ region of Myc (Fig. 2). The detailed structure of Myc–Max may give rise to novel therapeutic strategies that interfere with either the formation of the tetramer or the association with Myc CTD interactors.

2.2. Myc interaction with chromatin remodeling proteins

In addition to the well-documented association with Max, Myc has been shown to interact with a number of additional transcription factors and co-factors that modulate its activity.

The DNA bound Myc–Max heterodimer interacts through the Myc N-terminal region with a variety of proteins involved in transcription. TRRAP is of particular interest as it forms part of a multiprotein complex with histone acetyl-transferase (HAT) activity. Myc contributes to chromatin remodeling through an MBII-dependent interaction with TRRAP [40,41]. Inhibition of TRRAP synthesis or function blocks Myc-mediated oncogenesis, establishing an essential role for TRRAP in Myc activity [42]. The yeast homologue of TRRAP, tra1 is a component of the SAGA (SPT/ADA/GCN5/Acetyltransferase) complex [43] which also contains the HAT GCN5. The human homologue, hGCN5, has been shown to interact in a complex with Myc through TRRAP [44]. A Myc-Gcn5 fusion protein can partially restore the ability of a Myc MBII deletion mutant to induce transformation, suggesting that recruitment of HAT activity is an important component of Myc function. Myc also interacts with the p400 complex containing TRRAP, surprisingly this complex lacks HAT activity suggesting that Myc-TRRAP interaction may serve additional roles in addition to recruiting HATs. Nevertheless, p400 function in Myc-mediated oncogenesis remains unclear [45]. The recent discovery that CBP (CREB binding protein) binds to the Myc CTD provides an additional link between Myc CTD and activation of transcription. CBP functions partly through its HAT activity and was shown to interact with Myc *in vivo* and to stimulate Myc dependent transactivation. Interestingly, CBP also acetylates Myc *in vitro* and co-expression of the two proteins resulted in stabilisation of Myc [46]. Functionally, the biological significance of this interaction, in particular for Myc-mediated transformation, is yet to be determined.

Myc interacts with several other proteins implicated in chromatin remodeling, namely TIP48 and TIP49 [47]. Interaction of these molecules with Myc requires the Myc NTD and occurs independently of TRRAP binding. TIP48 and TIP49 have ATP hydrolysing activity, as well as suspected helicase activity and have been shown to be required for the foci formation by Myc and Ras in a primary co-transformation assay [47].

Myc may also be involved in a second mechanism of chromatin remodeling: an ATP-dependent process involving the recruitment of the SWI/SNF complex, which regulates transcription through nucleosome repositioning. Myc directly interacts with INI1/hSNF5, a key component of the SWI/SNF complex, and this was suggested to stimulate Myc transcriptional activity [48]. INI1/hSNF5 appears to be a tumour suppressor, and is mutated in the majority of atypical teratoid and malignant rhabdoid tumours [49–51]. More recently, Myc has been shown to interact with BAF53, an actin-related protein which is another integral component of the SWI/SNF chromatin remodeling complex. Targeted mutations in BAF53 inhibit oncogenic transformation by Myc [52]. In that context, it will be important to determine if other proteins of the SWI/SNF complex have the potential to interact with Myc and whether SWI/SNF recruitment may play a role in Myc-mediated transformation. Despite this enormous progress, many questions remain. Which genes are targeted by each of these distinct complexes? Does each complex drive a specific genetic program, such as transformation or apoptosis? Clearly, the precise role of these ATPase/helicase family proteins in Myc biology requires further study and this knowledge will be instructive in determining which complex is of highest priority for therapeutic targeting to block Myc transforming function.

2.3. Interactions with other transcriptional regulators

Whereas TRRAP appears to be a positive regulator of Myc-mediated transformation, Bin-1 (Bridging protein-1) appears to be a negative regulator [53]. The mechanism by which Bin-1 represses transformation is not fully understood, but it includes effects on the cell cycle as well as the promotion of apoptosis in response to Myc over-expression [54]. Since Bin-1 is deleted in a variety of tumours we sought to better define its interaction with Myc. Our group has used nuclear magnetic resonance (NMR) spectroscopy and biochemical assays to define the mechanism of interaction between Bin-1 and the MYC NTD [55]. We showed that a small proline-rich peptide within the conserved MBI interacts with the SH3 domain of Bin1 and that this interaction can be disrupted by phosphorylation of Myc Ser62. Our findings raise the intriguing possibility that the conserved MBI region may bind to other SH3 domain proteins. In addition, the data highlights the significance of post-translational modifications to Myc activity and suggests their modulation as an opportunity for therapeutic intervention.

Due to the evidence that Myc NTD binding proteins are critical to Myc function, we developed a novel high throughput screen termed the repressed transactivator assay (RTA) [56]. This two hybrid approach enables the full Myc NTD to be used as bait in the context of Myc as a transactivator bound to DNA. Novel methods, such as the RTA, will greatly facilitate the identification of Myc-binding proteins and inhibitors to disrupt these interactions. Using the RTA we screened a library derived from medulloblastoma cells with the Myc NTD as bait. A novel Myc interactor, JPO2, was isolated and shown to be a transcription factor containing a putative

LZ and a ring finger domain. JPO2 is closely related to a Myc transcriptional target, JPO1, and was also recently identified as a novel member of this emerging JPO family [57–59]. We show that JPO2 expression affects anchorage independent growth and is associated with metastasis in medulloblastomas [57]. As we have also observed Myc interaction with JPO1 protein, an interesting working model at a molecular level that emerges, sees Myc inducing JPO1 transcription for the formation of a Myc–JPO complex as a positive feedback loop to regulate gene transcription. It will be interesting to determine the validity of this model for the JPO family and to determine if additional Myc induced genes can also form functional transcription regulatory complexes with Myc.

Another novel Myc NTD binding protein is Krim-1, a nuclear zinc finger protein that contains a KRAB domain. Krim-1 was identified using the Ras Recruitment System (RRS) and was shown to associate with MBII [60,61]. In a reporter assay, Krim-1 was able to negatively regulate Myc transactivation and inhibit its oncogenic activity in REFs, a phenotype that is reduced in the presence of a Myc-binding mutant of Krim-1 [61].

Another interactor is the ARF tumour suppressor protein, which binds directly to Myc and inhibits its transcriptional activity in a p53-independent fashion. ARF blocks Myc ability to activate transcription without affecting its ability to repress transcription. ARF prevents Myc-induced transformation whereas Myc-induced apoptosis remains intact even in the absence of p53. ARF was shown to bind both Myc NTD and HLH-LZ domains of Myc [62,63]. These findings suggest a safeguard mechanism for preventing aberrant Myc signalling. The question naturally follows: does ARF differentially bind to a cohort of Myc-target genes related to proliferation and/or transformation? Studies using specific inhibitors of the Myc-ARF interaction will provide a direct link between ARF suppression of tumourigenesis and the control of Myc transcriptional activities. These findings highlight the critical role of MBII in transcriptional regulation and suggest that complexes, such as TRRAP, may either compete or cooperate with other multiprotein complexes for binding with ARF or Krim1.

Recently, several additional Myc-binding proteins have been described, but their role in transformation remains unclear. For example, Myc and PML were shown to co-localise within discrete nuclear structures associated with the nuclear matrix, termed PML bodies [64,65]. These PML bodies associate with regions of high transcriptional activity in the genome and have been implicated in diverse cellular processes, although their specific function remains open [66]. The differential effect that PML appears to have on Myc activation is also interesting. In PML-null mouse embryonic fibroblasts (MEFs) the expression levels of numerous Myc target genes is altered [64,65]. Although PML was demonstrated to physically interact with the Myc NTD and is recruited at Myc regulated promoters, the exact mechanism by which PML influences Myc activity remains unclear. Further insight into the precise contribution of these interactors, as well as others such as PARP-10 [67] or human papillomavirus E6 [68], to the function of oncogenic Myc in the carcinogenic process will determine whether targeting these proteins may be of merit.

2.4. Interactors that regulate Myc stability

Multiple mechanisms ensure proper control of Myc activity in normal cells, including regulation of Myc protein turnover, through the ubiquitin-proteosome pathway [69,70]. Recently, a component of the E3 ubiquitin ligase complex, Skp2, has been reported to mediate Myc turnover *in vivo,* which in turn is linked to Myc transcriptional activity at specific target genes. The regions of Skp2–Myc interaction were mapped to MBII as well as to Myc CTD (Fig. 2) [71,72]. Consistent with its role as a co-factor for Myc, Skp2 induces S-phase entry in a Myc-dependent manner [71]. Since Skp2 is known to have oncogenic properties [73,74] its role in increasing Myc transcriptional activity is not surprising. Although Skp2 is required for the phosphorylation-dependent degradation of various proteins, such as the tumour suppressor FOXO1 [75], there is no evidence suggesting the effect of Skp2 on Myc is regulated at the level of phosphorylation. Phosphorylation-dependent degradation of Myc involves two key residues, T58 and S62 within the MBI. Phosphorylation of S62 is mediated by Ras signalling and is a prerequisite to the phosphorylation of T58 that is performed by glycogen synthase kinase 3 (GSK3) [70,76–78]. Phosphorylation at T58 destabilises Myc protein [70,79]. T58 represents a major hotspot for mutations in Burkitt's and other lymphomas [80]. T58-phosphorylated Myc is specifically bound by the F-box protein, Fbw7, and results in Myc ubiquitination and degradation [80–83]. Fbw7 appears to function as a tumour suppressor gene [84]. Knockdown of Fbw7 increased both the abundance and transactivation activity of endogenous Myc. Surprisingly, one of the Fbw7 isoforms (Fbw7γ) co-localises with Myc in the nucleolus upon proteosome inhibition suggesting that Myc is also regulated in this specialised nuclear compartment for degradation [81]. These data support the idea that Fbw7 and Skp2 have opposing effects on Myc activity by targeting MBI and MBII, respectively. This knowledge can be exploited for therapeutics as targeting MBI might affect Myc stability while targeting MBII would affect Myc transcriptional activity.

3. Myc regulated target genes

To understand the role of Myc in cellular physiology and pathology, it is essential to identify the *bona fide* target genes regulated by Myc. A *bona fide* Myc-target gene is one whose regulatory region is bound, directly or indirectly by Myc, and whose expression is then regulated under an appropriate stimulus. These targets are distinguished from the many regulatory events that occur as a downstream consequence of Myc activity, like cell cycle progression [11]. In the past, many criteria were required to distinguish a gene as a true Myc target gene, but none were absolute. Several cDNA microarray studies have identified many *bona fide* targets and downstream regulated genes, but rarely have the two subsets been clearly distinguished [85–88]. Recently developed techniques have revolutionised this major issue and allowed true target genes to be rapidly identified and profiled *in vivo*, in any given cell or tissue under any given stimulus. One such technique is termed ChIP–chip (or ChIP-on-chip), in which the sensitivity and specificity of chromatin immunoprecipitation (ChIP) is combined with the high throughput capability of microarray (chip) technology.

3.1. Genome-wide Myc binding profile

Knowledge of binding sites found within a genome is essential for understanding the target genes regulated by any transcription factor. With the development of high throughput ChIP technologies, many labs, including our own, have identified the *in vivo* genomic DNA-binding sites of Myc [29]. This is especially important for understanding the mechanisms of Myc role in carcinogenesis. By profiling the many target genes regulated by Myc, the anticipation is that a key subset essential for a particular biological function will be distinguished and the mechanism of co-regulation of this cohort of genes will then be determined. Several novel insights have been forthcoming from these studies on Myc genomic binding in mammals [89–91]. It has been concluded that at sites of transcriptional activation, Myc and Max bind together at high affinity loci containing canonical or non-canonical E-boxes, which are often CpG rich [29,92]. Moreover, these characteristics are evolutionarily conserved from *Drosophila* to mammals [93]. In addition, recent work shows that there is evolutionary conservation of promoter architecture across different species of *Drosophila* containing a single E-box located within the first 100 nucleotides downstream of the transcription start site [94]. These outcomes suggest that a subset of Myc targets share a common and particular mechanism of regulation. By contrast, the repression of the target genes by Myc does not occur at E-boxes, but, rather at proximal promoter regions [95–98]. Interestingly, the Myc–Max interaction is essential for Myc to repress as well as activate gene transcription [91]. A systematic analysis across promoters, employing short oligonucleotide arrays, showed that only 22% of the Myc binding sites are located at the 5′ upstream region of protein-coding genes, while 36% are placed at the 3′ end of well-characterised genes that are associated with non-coding RNAs [99]. In light of this study it is very interesting that two recent papers describe the relationship between Myc and non-coding microRNAs (miRNAs). The first shows that Myc regulates a cluster of miRNAs in chromosome 13 that influences the major cell cycle regulator, E2F1 [100]. The authors proposed that Myc directly binds to this miRNA cluster to negatively regulate E2F1, thereby dampening the runaway effect of Myc inducing E2F1 transcription. It will be interesting to determine the function of this miRNA cluster in tumour cells. The second study shows that another cluster in chromosome 13, which is often overexpressed in lymphomas, strongly cooperates with Myc in lymphomagenesis by inhibiting Myc apoptotic capability [101]. The identification of miRNAs is in its infancy and the identification of transcriptionally active sites by ChIP–chip will greatly aid in delineating oncogenically active miRNAs.

One of the most profound results emanating from the genome-wide localisation studies is that Myc binds to an enormous number of target genes, compared with other transcription factors such as p53 and Sp1 [99]. Moreover, the genomic binding sites are associated with genes whose products are engaged in a wide-range of biological processes. In addition, it is curious that not all Myc bound targets are regulated at the level of expression [89].These observations have been gathered from a limited series of experiments conducted in a variety of labs. Clearly, it will be critical to systematically evaluate the nature of Myc target genes and their regulation under a multiplicity of physiological and pathological conditions. Importantly, these experiments are now feasible thanks to the advances in ChIP–chip technologies. To take advantage of these results, it will be necessary to identify Myc interactors that cooperate in the regulation of genes implicated in Myc related phenotypes. The target genes identified to date are compiled in a well-annotated database http://www.myc-cancer-gene.org/site/mycTargetDB.asp [11,102,103]. Due to space constraints, we will only discuss specific *bona fide* targets in the context of understanding their mechanism of regulation by Myc.

3.2. Mechanisms of Myc dependent transcriptional activation

Activation of target genes by Myc involves at least two regulatory steps; chromatin remodeling and promoter clearance. Mechanistic analyses of Myc-induced genes have clearly shown that Myc participates in chro-

matin remodeling when recruited to promoter regions. For example, the activation of the normally silent telomerase reverse transcriptase gene (TERT) by oncogenic Myc in exponentially growing fibroblasts requires TRRAP recruitment and is accompanied by both H3 and H4 acetylation [104]. Despite the profound role of this target gene in oncogenesis, transformation of primary cells is thought to require the regulation of additional targets by oncogenic Myc, as TERT overexpression alone cannot replace Myc in Rat fibroblast transformation assays [105] and mice lacking telomerase RNA can still be transformed by Myc and H-Ras [106]. Many cell cycle components are also regulated by Myc in a MBII/TRRAP-dependent manner [107]. Myc stimulates expression of the Cyclin D2 and Cdk4 genes, leading to sequestration of the cell cycle inhibitor p27 in CyclinD2/Cdk4 complexes [108–110]. Remarkably, p27 is then degraded by the protein product of two Myc activated genes, Cul1 and Cks [109,111,112]. Evidence suggests that the interaction of Myc-TRRAP to recruit HAT activity to target gene promoters such as TERT and Cyclin D2, is important for Myc transformation and may be dispensable for Myc to drive apoptosis [42,44]. For example, MEFs derived from the Cyclin D1/D2 double knock-out mice showed impaired proliferation in response to ectopic Myc expression, but the ability of Myc to potentiate apoptosis remained intact [113].

In addition to directly regulating chromatin remodeling through the recruitment of HATs, Myc also induces target genes involved in the regulation of chromatin dynamics. Recently, two such target genes have been described. MT-MC1 encodes a nuclear protein with homology to certain DNA helicases, and HMG-I encodes one of the high-mobility group proteins. Interestingly, ectopic expression of these proteins in Myc knock-out rat fibroblasts was shown to reconstitute many Myc phenotypes, such as rescuing the parental cell morphology, correction of the slow growth rate, cell size, genomic instability, clonogenicity, tumourigenicity and the regulation of a subset of Myc target genes [114]. Even though these two genes sometimes differ in the reconstitution of Myc functions, significant overlap exists between them. Remarkably, these chromatin remodeling proteins, either individually or in combination, were not able to complement the ability of Myc to potentiate apoptosis following serum withdrawal. These studies leave the door open to a selective investigation of additional fundamental Myc targets, which can restore, for example, the Myc apoptotic function.

Myc has been shown to participate in the transcription regulatory step associated with promoter clearance of RNA polymerase II (RNAP II) [115]. Myc was previously shown to recruit components of the elongation factor P-TEFb (positive transcription elongation factor b) at the *cad* promoter [115,116]. Inhibition of the P-TEFb complex blocked the effects of Myc in transformed cells [117]. Thus, the rate-limiting step of transcriptional activation of specific target genes may be Myc-mediated recruitment of P-TEFb, which then allows phosphorylation of RNAP II and release of a suspended transcription complex. It will be interesting to determine whether a cohort of genes, like *cad*, is similarly regulated by Myc and involved in the control of a common biological activity.

An attractive new development in understanding Myc function as a regulator of gene transcription is the appreciation that Myc not only regulates RNAP II regulated genes, but also affects genes transcribed by RNAP I and III. In fact, the various components of the ribosomal machinery are synthesised by all three RNA polymerases, RNAP I, II and III (Fig. 3). This is consistent with the regulation of ribosomal targets as a common feature of many expression microarray analyses to identify Myc target genes. Ribosomal biogenesis is a fundamental cellular process that takes place in the nucleolus and is essential for ribosome assembly and protein synthesis. Remarkably, the nucleoli are enlarged in cancer cells and several ribosomal proteins are overexpressed in tumours, suggesting a correlation between the deregulation of protein biosynthesis and cancer, leading to an opportunity for the development of innovative therapeutics targeting the translation machine [26]. Mechanistically, Myc binds to TFIIIB, a component of RNAP III machinery, and stimulates RNAP III transcriptional regulation of the 5S rRNA gene [118,119]. In the nucleoli, Myc directly regulates RNAP I transcription by interacting with SL1 (TIF-IB), an essential complex composed of the TATA binding protein (TBP) and three RNAP I-specific TBP-associated factors (TAFs) [120,121]. Additionally, ChIP experiments show that Myc and Max bind at non-canonical E-box sequences located within ribosomal DNA (rDNA) promoters. This association is followed by recruitment of TRRAP, enhanced histone acetylation, recruitment of RNAP I, and activation of rDNA transcription [122–124]. Taken together, regulation of all three RNA polymerases suggest that Myc plays a key role within the cell to produce molecules implicated in ribosome biogenesis (Fig. 3).

3.3. Mechanisms of Myc dependent transcriptional repression

The molecular mechanism of Myc role in repression of gene transcription remains less well characterised than its role in activation, yet all indications suggest repression is as important as activation for Myc function. For example, structure–function analyses have linked transformation to repression. Moreover, microarray and ChIP–chip analyses show that Myc activates and represses gene expression in similar proportions.

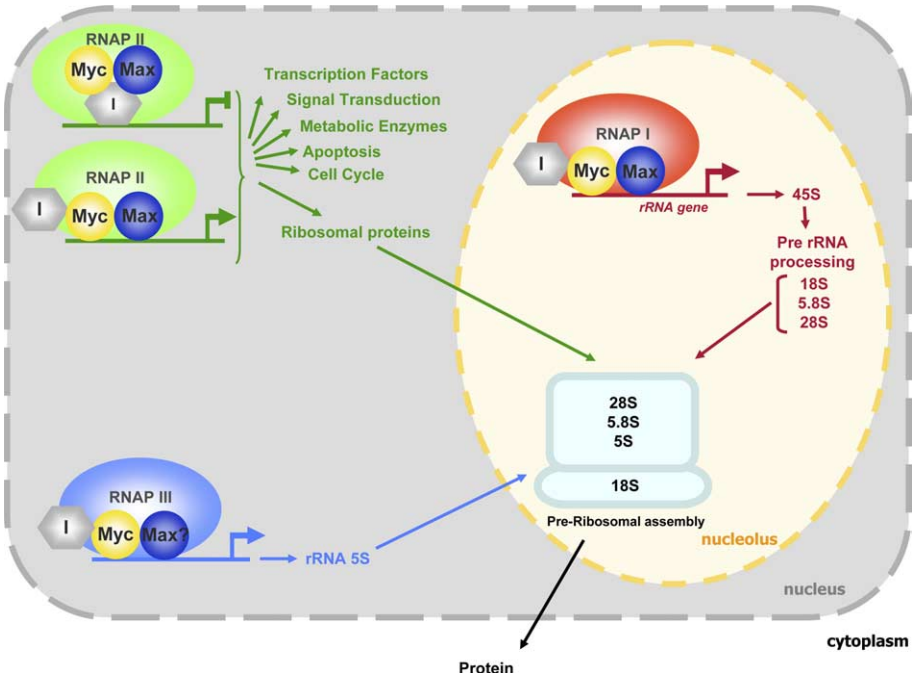

Fig. 3. Myc regulates transcription directed by the three RNA polymerases. Myc, with its partner Max, binds DNA and either activates or represses transcription of numerous target genes of enormous diversity, which are regulated by RNA polymerase II (RNAP II) and implicated in a multiplicity of cellular functions. In the nucleolus, together with Max as a partner, Myc activates the transcription of the rRNA genes, regulated by the RNA polymerase I complex (RNAP I). As well, Myc activates rRNA 5S transcription by interacting with TFIIIB, a component of the RNA polymerase III complex (RNAP III). The collective regulation of these three transcriptional functions by Myc suggests that Myc plays a key role within the cell to produce molecules implicated in ribosome biogenesis. RNAP I, II and III exist as a part of multiprotein complexes: other components are not shown in this simplified figure. The hexagon symbolizes various Myc-protein interactors (I) that contribute to gene regulation. (Figure adapted from Oskarsson and Trumpp [199]).

The target genes repressed by Myc fall into several functional categories. At a molecular level the best characterised targets includes those whose products inhibit cell proliferation (e.g. p15, p21, gadd45). Characterising the mechanism of Myc repression of these and other repressed target genes has shown that Myc does not appear to directly associate with the regulatory regions of repressed targets, but rather is recruited to core promoters through protein–protein interactions. Myc interacts with activating transcription factors, such as TFII-I, NF-Y and Miz-1 [95,125,126]. For example, transcriptional activation by Miz1 is abolished with Myc binding, and the Myc–Miz-1 complex acts as a transcriptional repressor; in part due to competition between p300 and Myc for binding to Miz1 [98]. It was recently shown that Myc represses transcription of p21Cip through recruitment of the DNA methyltransferase corepressor Dnmt3a. Myc and Dnmt3a form a ternary complex with Miz-1 to corepress p21Cip [127]. The precise role of Miz-1, as well as other factors including YY1 [128,129], NF-Y [95,130] and TFII-I [126] in Myc repression remains unclear and will require additional analyses.

Like Myc activation of gene transcription, Myc repression can occur through multiple mechanisms. Myc repression was thought to be dependent upon an initiator (Inr) region within the promoter, however, the presence of an Inr is not essential as genes lacking an Inr, such as gadd45 and PDGFB, are repressed by Myc [95,131]. Myc has been shown to repress gadd45 by a post-RNA polymerase II recruitment mechanism [132]. Interestingly, promoter binding and repression of PDGFBR are separable activities, since mutant Myc proteins that are unable to repress PDGFBR gene expression, still bind to the promoter *in vivo* [130].

Currently a large number of *in vivo* Myc activated or repressed target genes have been compiled from studies in different types of cancer cells. However, many questions remain. For example, which target genes are regulated by Myc and critical for the carcinogenic process? This issue will be essential to further study the subset of Myc regulated genes that directly or indirectly contribute to the tumour formation. Through the characterisation of these target genes, new possibilities will be addressed in the diagnostic field as well as in the development of novel anticancer therapeutics to target the oncogenic activity of Myc.

4. Myc as a target in cancer therapy

Developing therapeutics to inhibit oncogenic Myc would have enormous impact on the treatment of a

wide-range of human cancers. Transgenic mouse models provide a glimpse of how profoundly effective blocking Myc can be as an anti-cancer target *in vivo*. A series of elegant experiments using inducible Myc in hematopoetic cells [133], mammary gland [134], liver [135], skin [135,136] and pancreatic islets [137] have demonstrated that induction of oncogenic Myc leads to full-blown malignancies, while blocking Myc activation in most cases results in tumour regression [138]. This strongly supports the notion that targeting Myc in tumours represents a valid therapeutic approach. Caution should be exercised as not all tumours regressed upon withdrawal of Myc and a small proportion proceeded to hematological malignancies [133], while half of Myc-induced mammary carcinomas acquired mutations in K-Ras or H-Ras, thus rendering them independent of Myc status [134]. It is likely that anti-Myc agents would have to be coupled with therapies targeting Ras or other oncogenic pathways.

Many strategies are under development to target or exploit oncogenic Myc in tumour cells and eradicate the malignant cellular clones (Fig. 4). One approach is to disrupt Myc expression by targeting regulatory steps ranging from transcription to translation. Another strategy is to block Myc function by inhibiting critical protein–protein interactions that are essential for Myc to regulate gene transcription, such as heterodimerisation with Max. The working assumption is that targeting the Myc regulatory network will trigger the lethality of the tumour cell without causing irreversible damage to neighbouring normal cells. Such a tumour-specific effect is evident with other inhibitors that target universally expressed oncogenes. For example, antisense *bcl-2* will sensitise tumour cells to undergo apoptosis in response to low-dose chemotherapy, but non-transformed cells are spared [139–142]. In this case, the tumour cell has become dependent upon the deregulated signalling pathway and even marginal down-regulation of this lifeline renders it susceptible to extinction. Should this assumption be incorrect, Myc inhibitors will have to be targeted by tumour-specific delivery mechanisms. Newer strategies aim to achieve a high therapeutic index by targeting tumour-specific Myc-protein interactions and/or gene regulatory functions. Yet others aim to exploit the presence of oncogenic Myc expression in tumour cells to specifically trigger a suicide response exclusively in these transformed cells. Both ongoing and emerging strategies will be further discussed below.

4.1. Targeting Myc expression

One of the first successful applications of antisense technology targeted Myc expression, and through advancements over the last 15 years, this approach remains at the forefront of anti-Myc therapeutics [8,143]. Antisense oligonucleotides (ASOs) are short single-stranded DNA molecules that specifically target, hybridize and inhibit the mRNA of a selected gene (Fig. 4).

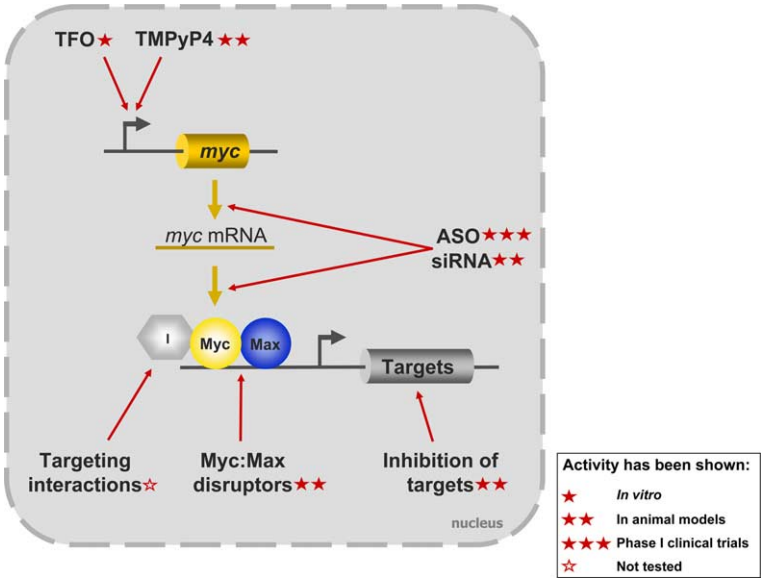

Fig. 4. Strategies for targeting oncogenic Myc in cancer. Therapeutic agents are coded according to their current therapeutic stage of development. Agents that have demonstrated efficacy *in vitro* are indicated with one star. These include inhibitors that block Myc expression, such as triple helix forming oligonucleotides (TFO). Double star agents have been tested in animal tumour models or xenograft models for their efficacy to inhibit Myc. This category includes disruptors of Myc–Max interaction and agents that block Myc target gene function, such as cationic porphyrins (TMPyP4) and small interfering RNAs (siRNA). Some of the antisense oligonucleotides (ASO) that have successfully completed Phase I clinical trials, and are at advanced stage of drug development are indicated by three stars. Finally, a clear star indicates additional potential targets for the therapeutic interference of Myc–protein interactors (I).

The DNA–RNA duplex then recruits RNase H endonuclease to cleave the RNA strand in the duplex [144], leaving the antisense DNA intact to hybridize to additional mRNAs of the target gene [145,146]. Antisense technology has evolved as a viable therapeutic alternative by increasing the functional stability and permeability of the ASOs. This has been largely achieved by replacing the phosphodiester backbone with a nuclease resistant phosphorothioate linkage (PS ASOs) [147,148].

Myc ASOs have advanced successfully through the many stages of preclinical evaluation and have demonstrated anti-cancer potential. Evaluation *in vitro* has shown that downregulating Myc expression by PS ASOs reduces leukaemic cell proliferation, induces differentiation and inhibits G1/S progression [149,150]. Moreover, exposure of MCF-7 breast cancer cells to Myc ASOs induced growth arrest [151]. In mouse models, treatment with Myc PS ASOs resulted in the delay or prevention of Burkitt's lymphoma [152–154] and Myc ASOs enhanced the efficacy of cisplatin to target melanoma both *in vitro* and *in vivo* [155]. Cisplatin resistance can be overcome by inhibiting Myc using AVI-4126, a phosphorodiamidate morpholino oligomer (PMO), in the Lewis lung carcinoma model [156]. AVI-4126 also inhibited growth of a murine prostate cancer xenograft by inducing growth inhibition and apoptosis *in vivo* [157]. These results have led to Phase I clinical trials, which show that intravenous administration of the morpholino oligomer was not accompanied by toxicity or serious adverse events, and importantly, the bioavailability was measurable in malignant tumours. The number of patients studied remains modest, yet this data supports the use of the AVI-4126 PMO as a potential therapeutic for cancer treatment [158]. It will be interesting to monitor the efficacy of this agent as it advances through to Phase II/III clinical trials.

Several additional Myc ASOs show promise for future application as anti-cancer agents. Administration of INX-6295, a 16-mer Myc PS ASO encapsulated in lipid particles, shows antitumour efficacy against a human melanoma xenograft. When administered with cisplatin, INX-6295 produced complete tumour regression in 30% of treated mice [159]. Furthermore, the combined application of *bcl-2* ASO/cisplatin/INX-6295 in mice harbouring human melanoma xenografts overexpressing either *bcl-2* or *c-myc* oncogenes resulted in effective antitumour therapy [160]. Yet another agent, the novel psoralen- or acridine-modified, clamp-forming ASOs, can downregulate Myc expression and synergise with cisplatin to inhibit melanoma cell proliferation and tumour progression [161,162]. The peptide nucleic acid (PNA) ASO also shows promise. PNA is a synthetic DNA in which the sugar-phosphate backbone is replaced with a polyamide-(2-aminoethyl) glycine-skeleton. This modification provides PNAs with a long half-life and enables PNAs to specifically hybridise to DNA and/or RNA in a complementary manner, forming a strong and effective duplex that can inhibit transcription and translation of the target gene [163]. Myc expression was rapidly downregulated in Burkitt's Lymphoma, using a 17-mer anti-*c-myc* PNA covalently linked to a nuclear localisation signal (NLS) (PNA-myc_{wt}-NLS) [164]. However, because this PNA blocked Myc in both transformed and non-transformed cells, further innovative refinements of this strategy are under development to increase tumour specificity [165]. Clearly, ASO technology targeting Myc expression is well advanced and has enormous promise for future application to patient care. Maximal efficacy will depend on achieving synergy with conventional chemotherapies and/or novel molecular anti-cancer agents [166,167].

RNA interference (RNAi) is a modern and popular approach to knockdown gene expression that has potential for drug development. RNAi is a mechanism for silencing gene expression through targeting double-stranded RNA to mRNA resulting in degradation of the targeted mRNA (Fig. 4). In mammalian cells, long double-strand RNAs are cleaved into small interfering RNAs (siRNAs) that, through incorporation into the silencing complex, direct target recognition. RNAi has been touted as the next major tool in targeted cancer therapy, because of its impressive specificity and efficacy. Compared to antisense approaches, siRNAs are 1000-fold more active [168] and a plethora of data shows the efficacy of siRNAs in cell culture. For example, Myc siRNA effectively inhibited Myc protein levels in MCF7 cells [169]. It appears that siRNA can be effective *in vivo* [170], but this has not yet been thoroughly explored. The primary limitations of siRNA *in vivo* remain its stability and delivery. siRNA is quickly degraded in plasma, so the duplex will need to be chemically modified for use as a drug. Perhaps strategies used to modify ASOs to increase stability can be exploited for siRNA. Recently the issue of delivery was addressed by fusing multiple siRNAs, targeting Myc, Hdm2 and VEGF mRNAs, to a positively charged protein that was covalently linked to a specific antibody. This ensured that only melanoma cells ectopically expressing that particular ligand were growth inhibited *in vivo* [171]. With this novel approach it may be possible to deliver such a lethal siRNA cocktail through a Myc-induced cell surface molecule to achieve tumour-specific targeting. Whether siRNAs target mutated oncogenes like Ras [172] and/or deregulated oncogenes like Myc [169], these new tools are likely to make a firm and lasting entrance into the arsenal of therapeutics in the fight against cancer.

Another series of agents have been designed to target *myc* expression at the transcriptional level. One promising agent includes the triple helix forming oligonucleotides (TFOs) which bind to double-stand purine-rich DNA within promoter regions and block transcription factor binding (Fig. 4). Phosphothioate stabilised TFOs

directed against the promoter region of c-*myc* have been shown to successfully inhibit Myc expression in several leukaemia and lymphoma cell lines leading to cellular growth arrest and apoptosis [173,174]. TFOs conjugated to the DNA-intercalating agent daunomycin specifically downregulated endogenous Myc in prostate and breast cancer cell lines [175]. Cationic porphyrin TMPyP4, which inhibits Myc transcription by blocking G quadruplexes, a DNA structure formed from G rich single-strand DNA during active transcription is another approach to target Myc [176] (Fig. 4). TMPyP4 inhibited the *in vitro* transcription of *myc* and decreased tumour growth rates in xenograft models [176]. The original use of cationic porphyrin was to block telomerase by stabilising the telomeric G rich single stand DNA overhangs into G quadruplexes [176]. New Myc-specific porphyrin analogues show promising results, suggesting that this approach has merit for further development [177,178]. Introduction of G quadruplex-forming oligonucleotides into Burkitt's lymphoma cells resulted in growth inhibition by sequestering the factors that normally bind to the native G quadruplexes formed at the Myc promoter [179]. It will be interesting to monitor the development of these various agents that target *myc* transcription for their ultimate use as therapeutics.

4.2. Targeting Myc–Max interaction

Breaking the Myc–Max bond would clearly destroy oncogenic Myc function and several strategies to dissolve this partnership are well underway (Fig. 4). Many of the approaches under development have been advanced because of our knowledge of the detailed structural biology of this protein–protein interaction and the essential residues involved. Molecular modeling and mutagenesis have been used to identify specific amino acids that alter the specificity of dimerisation [180]. Four amino acids within the LZ of Myc were sufficient for homodimerisation, as well as heterodimerisation with Myc and Max. Furthermore, a mutant protein termed Omomyc, which interferes with Myc binding to E-box elements, was able to inhibit colony formation in NIH3T3 cells [181]. Introduction of Omomyc in a Myc-induced skin tumourigenesis model in mice [182] inhibited Myc-induced papillomatosis, as well as restored the normal keratinocyte differentiation program and skin architecture, both of which are otherwise disrupted by Myc activation [183]. Importantly, the positive outcome of Omomyc expression was completely dependent on the presence of an oncogenic Myc and therefore may be of therapeutic value for the specific targeting of Myc-deregulated cells without affecting the surrounding normal cells.

Knowing the structural domains required for the Myc–Max interaction inspired a dominant-negative approach to disrupt this linkage. Myc mutants expressing only the b-HLH-LZ or HLH-LZ domain rapidly induced apoptosis in 3T3-L1 mouse fibroblasts [184]. This study provided the rationale to devise additional strategies to directly exploit Myc heterodimerisation for the design of novel therapeutics. One of the major challenges with this approach lies in achieving efficient delivery of the drug to the nucleus of tumour cells *in vivo*. To this end, a mutant peptide derived from the helix-1 region of Myc was linked to an internalisation sequence [185]. The fusion peptide interfered with the transcriptional activity of Myc leading to the inhibition of MCF-7 cell growth. The stability and activity of this peptidomimetic molecule was increased [186,187] and a variant of the original peptidomimetic has been synthesised and tested in mouse models. Interestingly, analysis of inhibitor interactions of Myc–Max shows the active molecules act through key basic residues at the outer surface of the Myc–Max heterodimer, potentially by binding or interfering with another interactor. The novel peptides were capable of reaching high concentrations in mouse organs and were effective at inhibiting growth of a colon cancer cell line. This peptide is 10-fold larger than traditional small molecules, which may contribute to its highly selective interference with Myc-specific protein–protein interactions [186]. It would be of great interest to test which specific Myc–protein interaction is inhibited *in vivo* and whether knockdown of this co-factor can directly trigger an anti-tumour effect in animals.

Recently, two groups have shown that inhibitors blocking the Myc–Max interaction can be isolated using a high throughput screen. In one study, inhibitors were identified by fluorescence resonance energy transfer in high-throughput screens of peptidomimetic libraries, then confirmed by enzyme-linked immunosorbent assay and electrophoretic mobility shift assay. The antagonists interfered with Myc-induced and Jun-induced oncogenic transformation suggesting the inhibitor may also target the LZ of the Jun oncoprotein [188]. The exact molecular mechanism of inhibition and the utility of these agents in the control of carcinogenesis is the focus of future analysis. In the second study, the yeast two-hybrid assay was used to screen a library of 10 000 compounds to identify those able to disrupt the interaction of the b-HLH-LZ regions of Myc and Max, yet show no general toxicity to the yeast [189]. Several compounds were identified and their ability to inhibit the Myc–Max interaction confirmed by an *in vitro* association assay. Using a reporter assay, the compounds were shown to inhibit Myc transcriptional activity and proved to inhibit growth of Myc transformed rat fibroblasts, but not Myc-null cells. Finally, incubation of transformed cells with the compounds for three days, prior to their injection into nude mice, inhibited tumour growth *in vivo*. Although the compounds used by both groups require relatively high concentrations, these promising studies

provide a platform for future development of more effective small molecule inhibitors of Myc–Max dimerisation.

5. Conclusions and perspectives

Targeting Myc at the level of expression and/or function is an effective approach to eliminate this potent oncoprotein. The agents described in this review successfully block Myc and have passed the first hurdle in targeted drug design. The issues that remain to be resolved include, but are not limited to, evaluating and improving sensitivity, specificity, delivery and efficacy as a single agent and in combination with other anti-neoplastic therapies. The agents that have advanced beyond Phase I clinical trials show enormous promise. It will be fascinating to monitor their progress and learn whether targeting Myc will have the expected impact at the level of patient care.

There are several additional approaches aimed at targeting Myc activity that are in the earliest stages of development. Based on the success of targeting Myc–Max interaction, there is significant interest in targeting other key Myc–protein partnerships, such as Myc-TRRAP. This field of study covers a broad spectrum of experimental analyses, from first identifying the interactor, determining whether it plays an important functional role in Myc transformation, through to mapping the precise points of interaction. Many Myc–protein interactions highlighted here have been described only in recent years and some of them may warrant inhibitor development.

A second major area of fundamental research that has the potential to impact therapeutic design aims at identifying and understanding Myc target genes and the biological pathways they regulate. As a multifunctional master regulator, Myc induces several genes which, in turn, play a critical role in transformation and are now being targeted as potential anti-cancer therapeutics. For example, haplo insufficiency for a Myc target gene, *odc* reduces skin tumourigenesis in mice [190] and a specific inhibitor of ODC, 2-difluoromethylornitive, can block Myc-induced oncogenesis [191]. Clearly, a greater understanding of the Myc transformation program will result in additional opportunities to target Myc function in tumour cells.

Another novel approach does not target Myc directly, but instead aims to exploit the ability of oncogenic Myc to potentiate apoptosis. A cooperating lesion, such as Bcl-2 activation, often inhibits Myc potentiation of apoptosis and collaborates with Myc to drive transformation. Blocking these anti-apoptotic molecules may release the ability of Myc to sensitise tumour cells to undergo apoptosis, elevate the therapeutic index and achieve tumour cell death. There is enormous effort to understand the nature of the genetic abnormalities associated with cancers that can block Myc-potentiation of apoptosis and contribute to transformation. With this knowledge, the innovative approach of exploiting deregulated Myc can be further advanced.

Myc deregulation is often associated with aggressive disease of poor prognosis, which augments the urgency for novel therapeutics targeting this potent oncoprotein. Many such agents are well along the drug development pipeline. Moreover, fundamental research instructs us daily of new opportunities to effectively target Myc expression and function to block malignant transformation. Further growth in the area of anti-Myc therapeutics is warranted and anticipated.

Conflict of interest statement

None declared.

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